Molecular Characterization of Drug Resistant Indigenous Methicillin Resistant *Staphylococcus aureus* (MRSA) Strains

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Abstract

Methicillin resistant *Staphylococcus aureus* (MRSA) complicates therapy of critical infections in hospitalsand livestock animals, globally. MRSA strains have also been escalated in community. The worldwide dissemination of MRSA (Hospital and Community acquired MRSA, and Livestock and food confined MRSA) with persistently varying drug resistance phenotypes and genotypes, and emerging novel molecular types is progressively being explored. Such continually modifying strains challenge the current therapeutic options. Therefore, it is crucial to survey regularly the prevalence of MRSA resistance patterns and its molecular types. MRSA strains (n=100) collected from various hospitals and diagnostic laboratories of Karachi, and their drug resistance patterns and MICs were found out and presence of *mecA* gene was confirmed by PCR. Literature was gathered from PubMed, Medline, PakMedinet, Google scholar and web of science etc. to detect the ratio of MRSA in hospitals, community, and livestock in Pakistan. The HA-MRSA was found with highest ratios (76%) in Lahore and CA-MRSA (57%) were recorded in Islamabad.All HA-MRSA were 100% resistant to Penicillin, Methicillin and Ampicillin and 90% Oxacillin-resistant, 87% Cefoxitin-resistant, 84% Amoxicillin-clavulanate-resistant, 77% Tobramycin-resistant, 71% Erythromycin and Ciprofloxacin-resistant, and 67% Norfloxacin-resistant. Increased MICs of Vancomycin, Teicoplanin and Linezolid has been recorded. Resistance genes and molecular types (*mec*A, *dfr*(B), *mec*C,*bla*Z, *gyr*A, *gyr*B, *grl*A, *grl*B, *rop*B, *aacA-aph*D1, *erm*(A), *erm*(C), *fos*(A), *vat*(C), *vat*(B), *tet*(K), *tet*(M), *agrI*, *agrII*, *agrIII*, and *SCCmec* types; I, II, III, IV, and VI) have been reported in many HA-MRSA. Present study confirmed the presence of *mecA* gene in 96% of HA-MRSA.

Key Words: MRSA, Hospital acquired-MRSA, Community acquired-MRSA, Livestock associated-MRSA, drug resistance

Tob Regul Sci.™ 2023;9(1): 971-994
DOI: doi.org/10.18001/TRS.9.1.67

Introduction

Staphylococcus aureus is placed in Staphylococcaceae family and is a spherical, coagulase and Gram reaction positive prokaryotic organism congregated likegrapes(Lakhundi and Zhang, 2018).It persistently settles in nasal area of animals and men, and externally over the body either as pathogen or harmless bacteria (Weese and van Duijkeren, 2010; Al-Amery et al., 2019).It has broad-ranged toxins and virulence determinants, commonly accountable for toxin-dependent illnesses; scalded skin syndrome, food reliant poisoning, and toxic shock syndrome (Okoli et al., 2018; Sadiq et al., 2020).Intrusive infections produced by methicillin-resistant *Staphylococcal aureus* (MRSA) are notable dilemma in health care centers and facilities, and healthy persons of community (Grundmann et al., 2010). The threatening of MRSA settlement in the body and infection has expanded from health care settings to community and animals (Lakhundi and Zhang, 2018).

The mobile genetic elements (MGE) harbored staphylococcal cassette chromosome (*SSCmec*) or specifically *mec*genes (*mecA, mecC*etc),are accountable for Methicillin resistant *S. aureus*(Ito et al., 2001). The evolution of MRSA is initiated by getting and inclusion of such MGEs into the genome of sensitive *S. aureus* (Lakhundi and Zhang, 2018).MRSA is liable for substantial ratio of hospital gained infections in Pakistan. Many investigations have intensely revealed the rising importance of clinical MRSA (Ashiq and Tareen, 1989; Siddiqui et al., 2017). Still, resistance is detected by phenotypic procedures and very finite information is accessible on molecular characterization of the clinical MRSA strains(Khan et al., 2020). MRSA strains carrying resistance to multiple antibioticsoffer a threat to relevant drug therapy and infection management team. So that, pursuing the prevalence and circulation of such resistant MRSA types is crucial (Archana et al., 2020). Nearly all strains of MRSA are MDR (multi-drug-resistant) and can only be contained by Vancomycin (a glycopeptide) but resistance (of low grade) against this drug has been disclosed(Rajaduraipandi et al., 2006).The PB2a is the product of *mecA*(Oliveira et al. 2006), which has insertion locations in plasmids and transposons helping the strain to resist multiple antibiotics.Therefore,MRSA strains are famous for having resistance against Lincomycin, macrolides, non-beta-lactams, Sulfamethoxazole, aminoglycosides, and quinolones (Chambers, 2001). Infections elicited by MRSA is not only reserved for men but also have been recorded in animals of farms andofdomestic value (Cattle, horse, goat, sheep etc.) (Saleha and Zunita 2010; Ullah et al., 2016).

1.1 Global prevalence, epidemiology and molecular types of MRSA strains

The ratio of occurrence and epidemiology of MRSA strains is regularly being modified with new and emerging MRSA strains arising in varied territories.Hence, consistent surveillance of MRSA strains by carefully checking the host range, typical features, and transmittance ways of novel types in each area is needed. Therefore, awareness of the epidemiology of MRSA at molecular grade is imperative in determining the current safety procedures and devising convenient prophylaxis (Lakhundi and Zhang, 2018).

In 1961, MRSA strain was initially recognized in United Kingdom, since then creating critical public health and hospital concerns(Patricia Jevons, 1961; Lowy, 1998).The presence of vancomycin-resistant-MRSA was witnessed in 2002 by Centers for Disease Control and prevention, USA (Graveland et al., 2011a). However, in the 1980s, similar strains begun to ariseand brought about the worldwide expansion (Sadiq et al., 2020). Despite the MRSA global circulation, its prevalence fluctuates in many countries. In Pakistan and India, MRSA occurrence ratio was escalated in contrast with European countries of north(Anwar et al., 2004). In 2016 and 2017, MRSA was noticed in the ratio around 48% in Peshawar and Karachi, Pakistan (Ullah et al., 2016; Rasool, et al., 2017).A report has witnessed the circulation of CC6-MRSA-IV, CC772-MRSA-V, CC8-MRSA-IV, and ST239-MRSA in the Middle East territory(Jamil et al., 2018).

For the classification of epidemiological attributes of MRSA types, it is necessary to apply related and authentic molecular approaches with highly differentiating capability to trace variations time to time. The ancestry and types of MRSA can be recognized by employing miscellaneous typing approaches at molecular level like *spa* and coagulase gene (*coa*), *SCCmec*, multilocus-sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE). The knowledge gathered from above techniques can be fruitful epidemiologically in discriminating between health care and community originated strains, determining the possible source of colonizing organisms (humans or livestock related), and tracking epidemics. Surely, some methods like PFGE are unable to trace out some MRSA strains (e.g., CC398), for this reason, a combo of the procedures may be required occasionally to typify few strains (Aires-De-Sousa et al., 2006; Bens et al., 2006; Cookson et al., 2007; Lakhundi and Zhang, 2018).Virulence determining markers e.g., *spa* and *coa* are straightly related to infection producing mechanism and strength.Both markers are highly variable and can present important understanding on strain variability (Sadiq et al., 2020).

Based on spa typing and MLST, 19492 and 6121 strains have been typified, respectively till July, 2020. Additionally, grouping of MRSA can be justified by *mecA*gene carried by *SCCmec* in to 11 (I-XI) varied types. Global occurrence of I to V types has been reported while other types of this classification prevailed in regional or local strains (Archana et al., 2020). The MRSA of community and hospital origin have peculiar molecular epidemiology and phenotypic characteristics. Usually,hospital-oriented MRSA strains are MDR carrying *SCCmec* types (I to III) however, community-oriented MRSA strains are non-beta-lactam sensitive and bear *SCCmec*type (IV/V) and produce Panton Valentine Leucocidin (PVL) (David and Daum, 2010).

In 2015, researchers had indicated the invasion of community-oriented MRSA strains into health care setups and reversed invasion had also been reported (health care to community). The global incidences of hospital-oriented MRSA strains (*SCCmec* type IV/V) and community-oriented MRSA strains (*SCCmec* type I to III) have been noticed (Bhutia et al., 2015; Dhawan et al., 2015). Various clones of hospital and community-oriented MRSA (sequence types; ST8,ST22, ST239, and ST772) were revealed with different prevalence in India under molecular epidemiology investigations. Archana and colleagueshave identified 60% and 40% hospital-oriented and community-oriented MRSA, respectively with around 23% Clindamycin and 7% Vancomycin intermediate resistance in India. Moreover, they found about 44% of hospital-oriented MRSA (harboring *SCCmec* IV and V) and 33% of community-oriented MRSA (*SCCmec* I and III) (Sunagar et al., 2016; Archana et al., 2020).

1.2 Clinical significance and merit of MRSA

MRSA elicited infections are culpable for more fatality rates than non-MRSA infections. Moreover, such infections contribute to prolong hospitalization and increased cost of treatment (Wolk et al., 2009; Antonanzas et al., 2015; Fortuin-de Smidt et al., 2015; Thampi et al., 2015). MRSA is liable to 25-50% of health care related infections and in the field of medicines;it poses a threat for containment and cure of relevant infections (Diekema et al., 2001). It also produces a great trouble because of large indices of illness and deaths and including its stabilization to beta-lactams (excluding Ceftobiprole and Ceftaroline) and allpresentpenicillins (Lakhundi and Zhang, 2018). Community-oriented MRSA are comparatively less resistant than hospital-oriented MRSA thus, can be cured particularly by employingfluoroquinolones, Clindamycin, Erythromycin, and aminoglycosides, etc. In contrast, hospital-oriented MRSA strain infected individuals need drugs effective against this strain. Since long time, Vancomycin has remained the only choice or last bullet for the cure of initial and precise option for critical MRSA infections. Furthermore, Vancomycin over practice has introduced Vancomycin-intermediate and resistant *S. aureus* (VISA and VRSA) strains in various territories of the earth (Tenover et al., 2001; CDC, 2002).

1.3 Community associated MRSA

The earliest case of Community associated MRSA strain was noticed in 1980s, since then its occurrence is on the verge(Elston, 2007), proposing the MRSA epidemiology has transformed with the worldwide rise of community associated MRSA types. Such strains are predominantly affiliated with the infections of soft tissues, skin and besides these other health care-related illnesses(Carleton et al., 2004; Donnio et al., 2004; Otter and French, 2011). They are somewhat genetically disparate than hospital strains of MRSA; less antibiotic resistant, frequently producing cytotoxin like Panton Valentine leucocidin (PVL) and having limited types of *SCCmec*(Pinho et al., 2001; Ma et al., 2002; Ito et al., 2003; Chambers and Deleo, 2009).Generally, community-oriented MRSA strains are confined to peoples of non-health care setups. Initially at the era of their rise, uncomplicated and temperate superficial and delicate tissue infections were noticed that was the principle of hospital-oriented and community-

oriented MRSA discrimination. However, now this scenario has been obscured as the occurrence of community associated MRSA strains has inflated. The disparity between these two kinds of strains has been inferiorly outlined because, many studies have endorsed the involvement of community-oriented MRSA as a main causative agent of health-care-associated outbreaks (Saiman et al., 2003; Healy et al., 2004; Bratu et al., 2005; Saunders et al., 2007; McAdams et al., 2008; Gregory et al., 2009; Maltezou et al., 2009; Teare et al., 2010; Nelson and Gallagher, 2012).

1.4 MRSA in livestock

A genetically diverse MRSA bearing Clonal Complex (cc398), wasinitiallyseparated from pigs of the France and Netherland, livestock of Europe, especially in broiler flocks, turkeys, pig herds and calves.Therefore, a new name livestock-associated MRSA (LA-MRSA) was established, taking the livestock as a distinct and novel MRSA reservoir (Larsen et al., 2012; Vossenkuhl et al., 2014).The MRSA of different genetic ancestry (containing ST9 sequence) is prevalent in the livestock of Asian countries (GüvenGökmen et al., 2018).Adaptation and infection of MRSA in various animals (companion, domestic, wild, aquatic etc.) have been disclosed(Cuny et al., 2010; van Duijkeren et al., 2010; Weese, 2010; Graveland et al., 2011; Dorado-Garcia et al., 2013). In Germany, MRSA strains have harmed 20% of cattle herds, pigs (>40%) and turkey (20-90%) (Kock et al., 2014; Idelevich et al., 2016). Various investigations have witnessed the great risk of LA-MRSA infection and colonization in individuals who are in close contact with animals. In Netherland, Swine farmers (23-32%) harbored LA-MRSA form pig farms. However, in Northern America this ratio is around 20%. According to these investigations, other animals and livestock may serve as constant sources of MRSA based illness in humans (Voss et al., 2005; van Rijen et al., 2008; Stein, 2009; Sadiq et al., 2020). However, the rising epidemics of LA-MRSA strains have been detected in hospitals and related infections are noticeably increasing in humans.For this reason, LA-MRSA has evolved as critical public health concern which requires strict check and control. It is believed that extensive application of antibacterials in the livestock, humans, and agriculture has imposed a selected pressure and has culminated the accelerated evolution and expansion of MRSA(Lewis et al., 2008; Pires et al., 2009). Further speedy dispersal of MRSA in livestock animals is supported by increased animal buying and selling, and overabundance in animal husbandry.Livestock-oriented strains of MRSA were also detected in uncooked meat of pork, veal, beef, and poultry obtained from wholesale market(Alt et al., 2011; Guo et al., 2018).Additionally, the colonization of such MRSA strains in various animals and in relevant handlers resulted by serial cross-contamination from butchery to processing, which is mostly connected with low grade or unhygienic measures taking during processing, slicing, improper storage, shipping and selling (Waters et al., 2011; Rinsky et al., 2013).

Entire world including Pakistan is facing the drug resistance dilemma in human and animals, and transmission of MRSA are led by weakened infection control and monitoring policies and persistently unchecked and uncontrolled contact of antibacterials to animals and humans.The after-effect of this problem has reduced the MRSA treatment choices so that, endless efforts to

monitor and contained MRSA is mandatory in all setups by evaluating the characteristics of evolving novel MRSA strains and their host range(Ali et al., 2018; Lakhundi and Zhang, 2018).

Materials and methods

In present investigation, MRSA (n=100) isolates of clinical specimens were obtainedfrom various hospitals and diagnostic labs of Karachi during 2019-2020 and were re-dentification by Gram-staining, colony features on blood agar, DNase agar, Mannitol salt agar, and coagulase test(ref). Confirmation of MRSA, drug susceptibility and MICs wereimplementedby pursuing the guidance of CLSI (CLSI, 2018). Erythromycin (E), Vancomycin (VA), Clindamycin (DA), Fusidic acid (FD), Amikacin (A), Co-trimoxazole (SXT), Ciprofloxacin (CIP) and Cefoxitin (FOX) disks were utilized. MICS of Vancomycin were ruled out by agar dilution method. DNA was extracted from MRSA (n=50) by boiling method. Molecular identificationof MRSA-specific gene, *mecA*was carried out by conventional PCR using forward and reverse primers (F=AAAATCGATGGTAAAGGTTGGC andR=AGTTCTGGAGTACCGGATTTGC). A 20µl PCR reaction volume was prepared by mixing 2µl of genomic DNA, 10µl of Master Mix (2x), 1µl of forward and 1µl reverse primers and 6µl sterile distilled water. Thermal cycler was adjusted to carry out PCR protocol as 95 °C for three minutes, thirty-three cycles of 94 °C for one minute, 53 °C for thirty seconds and 72 °C for one minute, and final elongation was carried out at 72 °C for six minutes. PCR products were analyzed 2% agarose gel electrophoresis (Pournajaf et al., 2014).

Literature was collected from different data bases including PubMed, Medline, PakMedinet, Google scholar and web of science. Local data was collected from direct search on google. The spot map of MRSA was generated by using epi info software version 7.2.4. Statistical analysis (t-test and standard deviation) was conducted by using Microsoft excel 2007.

Results and Discussions

Fifty-two research papers covering duration 2009-2021 were retrieved from various databases showing the MRSA (HA-MRSA, LA-MRSA, CA-MRSA and FA-MRSA) prevalence in various regions of Pakistan(Karachi, Lahore, Islamabad, Peshawar, Rawalpindi, Hyderabad, Rahim Yar Khan, Faisalabad, Abbottabad, Haripur, Kohat, Malakand, Interior Sindh and Baluchistan) shown in Fig.1. Accordingly in many cities of Punjab, the highest ratio (76%) of HA-MRSA occurrence was noticed in Lahore, 2020 (Saeed et al., 2020), whereas other ratios of HA-MRSA; 70%, 55%, 43%,33%, 32%, 15%, 13% and CA-MRSA (49%) were noted in 2019, 2014, 2014, 2020, 2018, 2017, 2020, and 2021, respectively (Fig. 1 and Table. 1) (Ahmad et al., 2014; Hassan, et al., 2014; Sohail and Latif, 2017; Iqbal et al., 2018; Tariq and Javed, 2019; Faiqa Arshad et al., 2020; Parveen et al., 2020; Sheikh et al., 2021). A total of 9 research articles stated the ratios of MRSA in Lahore (Fig. 1). In Islamabad,7 research manuscripts indicated the occurrence of MRSA (Fig. 1). Correspondingly in 2020, the highest prevalence ratios of HA-MRSA were recorded 65% (Table. 1) (Khan et al., 2020), followed by the 63% FA-MRSA in

2020 (Table. 2), 57% CA-MRSA in 2018 (Table. 2), 53% HA-MRSA in 2014, 48% HA-MRSA in 2021, 46% HA-MRSA in 2014, 43% HA-MRSA in 2018, and 20% HA-MRSAand 6% CA-MRSA in 2019 (Fig. 1)(Ahmed et al., 2014; Khan et al., 2014; Sohail and Latif, 2018; Abbasi et al., 2019; Sadiq et al., 2020; Rasheed et al., 2021). In Rawalpindi, 66% HA-MRSA (Table.1) and 33% CA-MRSA (Table. 2) were witnessed in 2019(Taj et al., 2019). Interestingly, the prevalence of CA-MRSA reached up to 50% (Fig. 1) (Mirza et al., 2020). In Rahim Yar Khan, HA-MRSA was found 67% in 2019 (Fig. 1 and Table. 1) (Hussain et al., 2019).LA-MRSA ratio in Faisalabad was found 34% in 2017 (Fig. 1 and Table. 2) (Aqib et al., 2017).

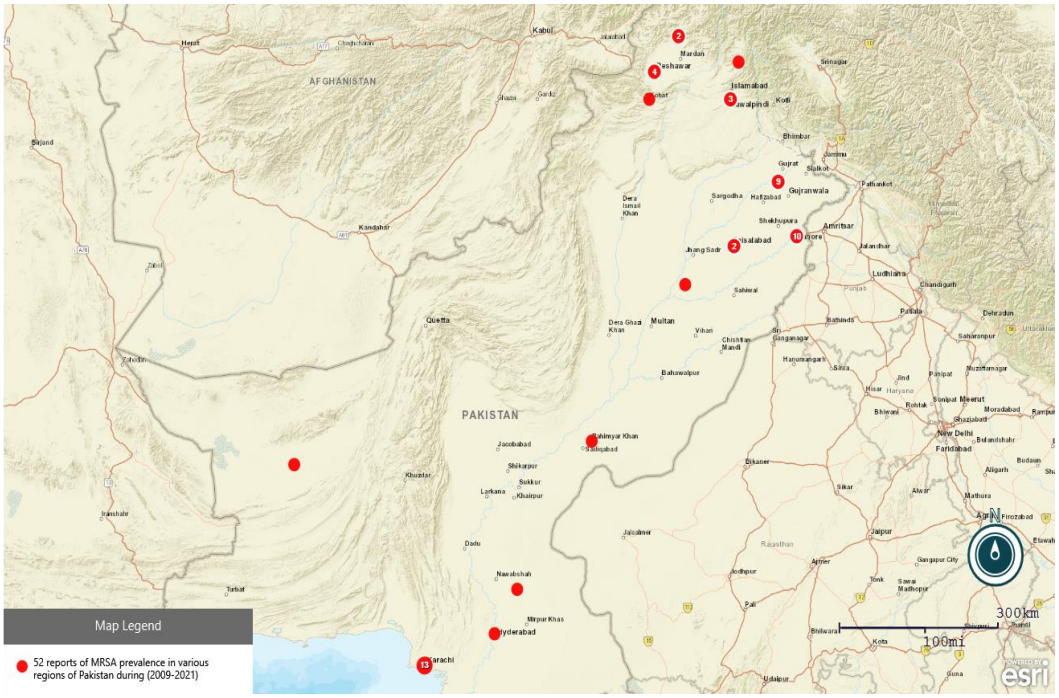


Fig. 1 Prevalence of MRSA reported in various cities of Pakistan

In Sindh, Karachi showed the highest percentage of HA-MRSA (71%), revealed in 2020 (Razzak et al., 2020) followed by 70% (present study), 66% (2019), 52% (2017), 50% (2017), 48% (2011 and 2017), 45% (2018), 39% (2010 and 2014), 30% (2014), 23% (2009), 15% (2013), and FA-MRSA (8%)in 2017 (Fig. 1,Table. 1 and 2)(Akhter et al., 2009; Taj et al., 2010; Ansari et al., 2011; Butt et al., 2013; Ahmed et al., 2014; Sabir et al., 2014; Merani et al., 2017; Rasool et al., 2017; Saleem et al., 2017; Tuba Siddiqui et al., 2017; Fatima et al., 2018; Hanif and Hassan, 2019). Thirteen research papers from Karachi were included. In 2012, 37% of HA-MRSA was recognized in Hyderabad (Fig. 1 and Table. 1) (Bano et al., 2012). In interior Sindh the ratio of HA-MRSA was 27% in 2017 (Fig. 1) (Saleem et al., 2017).In Baluchistan very limited data was available only one report represented the 4% of HA-MRSA in 2017 (Fig. 1) (Saleem et al., 2017).

In the province of Khyber Pakhtunkhwa, Peshawar city was observed for HA-MRSA prevalence as 54% in 2011, 36% in 2016, 27% in 2018, and 4.1% in 2015 (Fig. 1 and Table. 1) (Rafiq et

al., 2015; Ullah et al., 2016; Hussain et al., 2018). In Malakand division 44% prevalence of HA-MRSA was reported in 2016 (Fig. 1 and Table. 1) (Madzgalla et al., 2016).In Kohat, 44% of HA-MRSA was witnessed in 2013(Hussain et al., 2013), while Abbottabad 25% in 2015 (Fig.1 and Table. 1)(Taj et al., 2015). Interestingly, alarming ratio of LA-MRSA (87%) was found in Haripur, 2017 (Fig. 1 and Table. 2) (Syed et al., 2017).

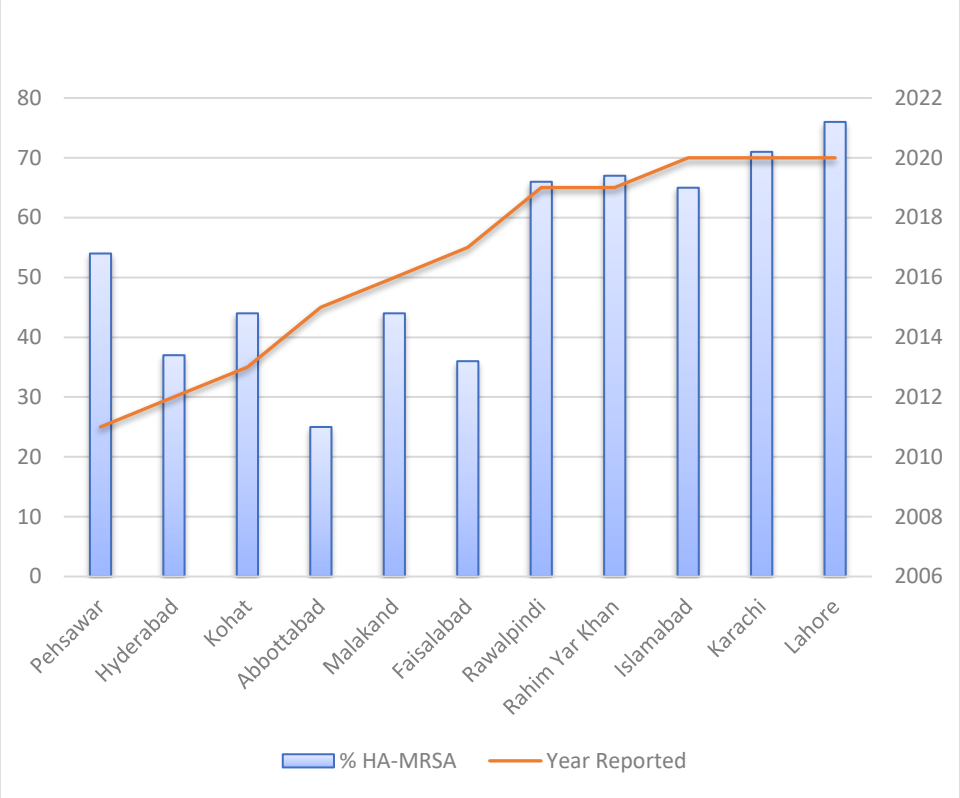


Fig 2. Comparison of prevalenceratios of HA-MRSA among various regions of Pakistan from 2011-2020

Highest prevalence ratios of HA-MRSA reported in various cities during 2011 to 2020 were chosen and compared (Fig. 2). Accordingly, Lahore showed the greatest ratio of HA-MRSA (76%) among all cities reported in 2020 (Saeed et al., 2020), followed by Karachi 71% in 2020, Rahim Yar Kahan 67% in 2019, Rawalpindi 66% in 2019, Islamabad 65% in 2020, Peshawar 54% in 2011, Kohat 44% in 2013, Malakand 44% in 2016, Hyderabad 37% in 2012, Faisalabad 36% in 2017, and Abbottabad 25% in 2015. Yearly increase in the appearance of HA-MRSA has been observed. High ratios of HA-MRSA in Lahore and Karachi may be because these are the large cities of Pakistan having crowded populations, increased inter-cities travelling of peoples for business or patients for treatment. The continuous progression in emergence of MRSA strains in livestock, community, food, and healthcare facilities is because of over, unnecessary, and illegitimate application of antibiotics in aforementioned areas producing the antibiotic selective pressure (Bano et al., 2012; Hussain et al., 2013; Taj et al., 2015; Madzgalla et al., 2016; Azhar et al., 2017; Hussain et al., 2019; Taj et al., 2019; Khan et al., 2020; Razzak et al., 2020).

City	HA-MRSA	Drug resistance	MICs	Drug resistance genes	Molecular types	Reference
Abbottabad	25%	AMX (100%), FOX (100%) MEM (92%), CEC (83%), CFR (83%), CIP (83%), E (83%), CAZ (79%), AMC (75%), CXM (71%), SCF (63%), ZOX (63%), CRO (50%), SXT (42%), CN (46%), IPM (0%)	MEM (512µg/ml), AMC (256µg/ml), AMX (256µg/ml), CAZ (256µg/ml), CXM (256µg/ml), SXT (256µg/ml), CFR (128µg/ml), CEC (128µg/ml), CIP (128µg/ml), E (128µg/ml), FOX (128µg/ml), IPM (128µg/ml), ZOX (128µg/ml), CN (64µg/ml), CRO (64µg/ml), SCF(64µg/ml),	Nd	Nd	Taj et al., 2015
Faisalabad	36%	LZD (48%), FOX (36%), VA (15%)	LZD (>4 µg/ml), VA (≥16 µg/ml)	*van*A (73%), *cfr* (78%)	Nd	Azhar et al., 2017
Hyderabad	37%	E (35%), DA (33%), OFX (31%), CIP (25%), TE (25%), AK (10%), FOS (4%), PIP (0%), VA (0%)	Nd	Nd	Nd	Bano et al., 2012
	Nd	MET (100%), OX (100%), E (99%),FOX (87%), CIP (36%), TE (36%), MXF (27%), OFX (16%), FD (12%), VA (11%), LZD (8%), RIF(7%), C (3%), CN (1%), LEV (3%), KAN (2%), MIN (2%), TEC (1%), DA (5%), Q/D (0%), TOB (0%)	Nd	*mec*A (20 strains)	Nd	Brohi and Noor, 2017
Islamabad	65%	CN (64%), E (50%) TE (36%), DA (26%), SXT (26%), RIF (20%), C (12%), Q/D (5%), LZD (4%)	CN (512 to >512mg/L), CIP (64 to >512mg/L), E (128 to >512mg/L), FOX (64 to>512mg/L), FOS (256-512mg/L), RIF (64-512mg/L), TE (128 to >512mg/L), VA (4-8mg/L, few strains),	*dfr*(B) (80%), *tet*(K) (87%), *tet*(M) (59%), *mec*A (54%), *mec*C (3%), *bla*Z (100%), *gyr*A (95%), *gyr*B (33%), *grl*A (9%), *grl*B (6%), *rop*B (80%), *aacph*D1 (75%), *erm*(A) (13%), *erm*(C) (100%) *fos* (A) ()81%, *vat* (C) (56%), *vat*(B)(29%), *vat*(A)(4%)	MRSA *agrI* (22%), *agrII* (7%), *agrIII* (20%) *SCCmec* Types; I (14%), II (23%), III (86%), IV (40%), VI (23%)	Khan et al., 2020

City	HA-MRSA	Drug resistance	MICs	Drug resistance genes	Molecular types	Reference
Islamabad	43%	CIP (100%), OFX (100%), TOB (100%), CN (100%), SXT (100%%), AK (100%), DA (75%), DO (75%), AZT (50%), FD (21%), C (10%), LZD (0%), VA (0%)	Nd	Nd	Nd	Sohail and Latif, 2018
Islamabad	46%	P (98%), FOX (96%), FD (65%), E (35%), SXT (23%), LZD (21%), LEV (19%), TE (17%), C (15%), VA (6%), TGC (0%)	Nd	Nd	*agrI* (46%), *agrII* (4%), *agrIII* (29%), *agrIV* (0%),	Khan et al., 2014
Islamabad	Nd	Macrolides (78%), Fluoroquinolones (62%), DO (59%), RIF (38%), TE (36%), FD (35%), SXT (32%), TGC (9%), C (7%), MIN (6%), TEC (6%), Q/D (0%), LZD (0%), VA (0%)	Nd	Nd	Nd	Kaleem et al., 2010
Islamabad	Nd	CIP (87%), CN (87%), E (85%), TE (83%), NEO (59%), AK (57%), RIF (52%), FD (20%), C (5%), TGC (5%), MUP (2%), LIN (0%), VA (0%)	VA (0.5-6µg/ml)	Nd	CC8 (100%, n=60), CC30 (5%, n=3) *SCCmec* Types; types III (37%, n=22), IIIa (20%, n=12) and IV (43%, n=26), *ST239* (15%, n=9), *ST8* (5%, n=3) and *ST113* (3%, n=2)	Shabir et al., 2010
Karachi	71%	E (90%), CN (88%), CIP (81%), DA (65%), TE (20%), SXT (14%), RIF (7%)	Nd	Nd	Nd	Razzak et al., 2020
Karachi	66%	AMP (100%), FOX (100%), E (91%), OX (91%), FOS (79%), CIP (73%), S (73%), FD (64%), DA (55%), TE (48%), CN (45%), VA (15%)	Nd	Nd	Nd	Hanif and Hassan, 2019

City	HA-MRSA	Drug resistance	MICs	Drug resistance genes	Molecular types	Reference
Karachi	45%	P (100%), E (88%), CIP (80%), CN (67%), DA (66%), FD (49%), SXT (38%), AK (36%), LZD (0%), VA (0%)	Nd	Nd	Nd	Fatima et al., 2018
Karachi	48%	FOX (100%), CIP (90%), E (78%), SXT (45%), AK (38%), FD (30%), DA (30%), VA (0%)	Nd	Nd	Nd	Rasool et al., 2017
Karachi	52%	FOX (100%), OX (100%), NA (68%), E (65%), CFR (62%), CIP (48%), DA (46%), TE (45%), RIF (28%), TEC (25%), VA (25%), MIN (15%)	Nd	Nd	Nd	Tuba Siddiqui et al., 2017
Karachi	50%	SXT (61.5%), DA (42%), FOS (21%), RIF (10%), FD (3%) VA (0%), LZD (0%), TEC (0%), TGC (0%)	DA (≤0.25- ≥8 µg/ml), VA (≤0.5- ≥2 µg/ml), LZD (≤0.5-48 µg/ml), RIF (≤0.5-≥ 328 µg/ml), SXT (≤10- ≥328 µg/ml), TEC (≤0.5-48 µg/ml), TGC (≤0.12-0.58 µg/ml), FOS (≤8- ≥128 µg/ml), FD (≤0.5- ≥328 µg/ml)	Nd	Nd	Saleem et al., 2017
Karachi	39%	CFP (98%), E (98%), CLA (93%), C (93%), CIP (93%), FOS (83%), FD (83%), SXT (83%), SCF (81%), DA (76%), AK (67%), TZP (60%), DO (45%), IPM (19%), VA (0%),	Nd	Nd	Nd	Sabir et al., 2014
Karachi	15%	DO (95%), FD (95%), TOB (95%) AK (91%), VA (23%)	Nd	Nd	Nd	Butt et al., 2013
Karachi	Nd	FOX (100%), P (100%), C (95%), E (84%), OFX (84%), CN (79%), DA (72%), TE (72%), SXT (56%), AK (20%), FD (15%), TGC (0%), VA (0%)	Nd	Nd	Nd	Nizamuddin et al., 2011
Karachi	39%	P (100%), CXM (100%), DO (100%), ATM (100%), NA (100%), CN (97%), SXT (96%), C (93%), TOB (81%), OFX (72%), CIP (64%), AMX (57%), CTX (48%), CRO (45%), FOX (36%), CFR (37%), FOS (31%), CXM (24%), AK (17%), MEM (14%), VA (1%), LZD (0%)	VA (32µg/ml, n=1), VA (8µg/ml, n=2), VA (16µg/ml, n=2)	*mec*A gene (100%, n=174)	Nd	Taj et al., 2010

City	HA-MRSA	Drug resistance	MICs	Drug resistance genes	Molecular types	Reference
Karachi	Nd	TE (82%), DA (79%), SXT (59%), RIF (50%), C (10%), FD (9%)	Nd	Nd	Nd	Idrees et al., 2009
Karachi	23%	CFR (100%), CLX (100%), P (100%), SXT (95%), AMC (90%), E (70%), CHL (65%), CN (55%), CIP (30%), MEM 20%), VA (0%)	Nd	Nd	Nd	Akhter et al., 2009
Karachi	70%	FOX (100%), E (80%), CIP (72%), DA (55%), SXT (58%), FD (42%), AK (39%), VA (0%)	Vancomycin (0.5-2µg/ml)	*mec*A (96%, n=48)	Nd	Present study
Kohat	44%	AMX (100%), MET (100%) CTX (76%), OFX (74%), LEV (70%), CFR (69%), E (69%), CN (67%), NOF (64%), CIP (59%), SPF (59%), C (34%), VA (1%),	Nd	Nd	Nd	Hussain et al., 2013
Lahore	13%	Nd	VA (≥ 1.5µg/ml)	Nd	Nd	Faiqa Arshad et al., 2020
Lahore	76%	AMP (100%), FOX (100%), MET (100%), ETP (100%), TOB (95%), TE (92%), MXF (89%), VA (18%), TGC (1%)	Nd	*mec*A (33%, n=10/30), *van*A (30%, n=9/30)	Nd	Saeed et al., 2020
Lahore	70%	Nd	Nd	Nd	*spa*1 (12%), *spa*2 (81%), *spa*3 (3%), *spa*4 (3%)	Tariq and Javed, 2019
Lahore	32%	TE (98%) CIP (98%), SXT (63%), FD (58%), LZD (0%), VA (0%)	Nd	*mec*A (n= 41, 100%)	*luk*S/F-PV (n=21, 51%)	Iqbal et al., 2018
Lahore	15%	OFX (98%), CIP (97%), TOB (92%), E (88%), AZT (88%), CN (87%), SXT (72%), DA (70%), AK (67%), DO (45%), FD (22%)	VA (0.5-8µg/ml)	Nd	Nd	Sohail and Latif, 2017
Lahore	Nd	P (100%), CN (73%), SXT (68%), CIP (66%), E (51%), DA (44%), DO (41%), LZD (0%)	VA (2µg/ml, n=29, 71%), (1µg/ml, n=12, 29%), (≥ 8µg/ml, n=4, 9.75%)	Nd	Nd	Cheema et al., 2017
Lahore	55%	AMP (96%), OX (55%), AMX (49%), FD (33%), LZD (10%), VA (2%)	Nd	Nd	Nd	Hassan, et al., 2014

City	HA-MRSA	Drug resistance	MICs	Drug resistance genes	Molecular types	Reference
Malakand	44%	Nd	Nd	*mecA* (31%), *blaZ* (87%), *linA* (2%), *mpb*BM (42%), *msrA* (42%), *aacA-aphD* (29%), *aalD* (2%), *aphA*3(44%), *sat* (44%), *tetK* (7%)	*SCCmec* IV (16%), *SCCmec* V (29%), ST772-MRSA-V (PVL+), Bengal Bay clone (29%), CC6-MRSA-IV, WA MRSA-51 (11%), CC8-MRSA-IV (PVL+/ACME+), USA300 (2%), CC509-MRSA-IV (2%)	Madzgalla et al., 2016
Peshawar	36%	P (100%), FOX (100%), LZD (0%), VA (0%), RIF (19%), C (23%), DA (25%), MIN (33%), SXT (35%)	VA (0.5 to 8µg/ml)	*mecA* (36%)	Nd	Ullah et al., 2016
Peshawar	4%	OX (100%), MET (100%), FOX (100%), AMX (100%), CTX (100%), CFR (100%), AMC (88%), CIP (88%), NOF (69%), DO (63%), TE (63%), LEV (56%), OFX (56%), CN (56%), E (44%), C (38%), SPF (38%), DA (38%), SXT(13%), VA (6%), LZD (0%)	Nd	Nd	Nd	Rafiq et al., 2015
Peshawar	Nd	AMP (100%), FOX (100%), CAZ (94%), CFM (94%), CEC (94%), AMC (85%), FEP (85%), CFP (85%), SXT (76%), CN (69%), MEM (50%), AK (46%), CLA (44%), C (39%), DO (11%), RIF (6%),	FOX (>256µg/ml), CFR (>256µg/ml), FD (4µg/ml), VA (4µg/ml), LZD (8µg/ml), CIP (128µg/ml)	Nd	C1aaba (n=9), C1a4b (n=7), C1b (n=3), C2a (n=3), C1b (n=3), C2a (n=3), C1a5 (n=2), D (n=2), E1aaa (n=2), E1aab (n=2), G1 (n=2), H (n=2), I1 (n=2)	Ahmad et al., 2014
Rawalpindi	66%	CIP (77%), CN (64%), VA (1%)	Nd	Nd	Nd	Taj et al., 2019
Rahim Yar Khan	67%	AMC (80%), OFX (71%), FOX (67%), TE (58%), CIP (48%), E (46%), AK (40%), VA (0%)	Nd	Nd	Nd	Hussain et al., 2019

Keys: **HA-MRSA**=Hospital associated methicillin resistant *S. aureus*, **Nd**=Not determined, **AK**=Amikacin, **AMC**=Amoxicillin-clavulanate, **AMP**=Ampicillin, **AMX**=Amoxicillin, **ATM**=Aztreonam, **AZT**=Azithromycin, **C**=Chloramphenicol, **CAZ**=Ceftazidime, **CEC**=Cefaclor, **CFM**=Cefixime, **CFP**=Cefpirome, **CFR**=Cefradine, **CHL**= Chloromycetin, **CIP**=Ciprofloxacin, **CLA**=Clarithromycin, **CLX**= Cloxacillin, **CN**=Gentamicin, **CRO**=Ceftriaxone, **CTX**=Cefotaxime, **CXM**= Cefuroxime, **DA**=Clindamycin, **DO**=Doxycycline, **E**=Erythromycin, **ETP**=Ertapenem, **FD**=Fusidic acid, **FEP**=Cefepime, **FOS**=Fosfomycin, **FOX**=Cefoxitin, **IPM**=Imipenem, **KAN**=Kanamycin, **LEV**=Levofloxacin, **LIN**= Lincosamide, **LZD**=Linezolid, **MEM**=Meropenem, **MET**=Methicillin, **MIN**=Minocycline, **MXF**= Moxifloaxacin, **MUP**= Mupirocin, **NA**=Nalidixic acid, **NEO**= Neomycin, **NOF**= Norfloxacin, **OFX**=Ofloxacin, **OX**=Oxacillin, **P**= Penicillin, **PIP**=Pipemidic acid, **Q/D**= Quinupristin-dalfopristin, **RIF**=Rifampicin, **S**=Streptomycin, **SCF**=Cefoperazone-sulbactam, **SPF**=Sparfloxacin, **SXT**=Trimethoprim-sulfamethoxazole, **TE**=Tetracycline, **TEC**=Teicoplanin, **TGC**= Tigecycline, **TOB**=Tobramycin, **TZP**=Piperacillin-tazobactam, **VA**=Vancomycin, **ZOX**= Ceftizoxime

Table 1. Prevalence,drug resistance, and molecular types of HA-MRSA in various regions of Pakistan

City	MRSA	Drug resistance	MICs	Drug resistance genes	Molecular types	Reference
Faisalabad	LA-MRSA (34%)	AK (20%), CN (10%), LEV (10%), CIP (0%), LZD (0%), MXF (0%), SXT (0%)	Nd	*mecA* (34%)	Nd	Aqib et al., 2017
Haripur	LA-MRSA (87%)	Nd	Nd	*mecA* (87%), *aac-aph* (87%), *pvl* (87%)	ST8-t8645-MRSA-IV, associated with USA300; and ST772-t657-MRSA-IV and ST772-t8645-MRSA-IV, both characteristic of the Bengal Bay community-associated MRSA clone	Syed et al., 2017
Islamabad	FA-MRSA (63%)	CIP (100%), NEO (100%), MET (100%), TE (100%), CN (82%), VA (80%), E (76%), AMX (70%), FOX (63%), NOV (52%)	Nd	*mecA* (100%)	*coa* (54%), *spa* (36%)	Sadiq et al., 2020
Islamabad	CA-MRSA (57%)	CN (100%), OFX (100%), CIP (100%), TOB (97%), SXT (95%), AK (90%), DA (70%), AZT (68%), DO (50%), C (30%), FD (21%), LZD (0%), VA (0%)	Nd	Nd	*pcv* and *SCCmec* IV/V	Sohail and Latif, 2018
Karachi	FA-MRSA (8%)	Nd	Nd	Nd	*SCCmecA* IV (74%), *SCCmecA* type II (20%), *SCCmecA* type III (5.8%)	Merani et al., 2017
Rawalpindi	CA-MRSA (33%)	CIP (77%), CN (64%), VA (1%)	Nd	Nd	Nd	Taj et al., 2019

Keys: **NOV**=Novobiocin, **LA-MRSA**=Livestock-associated methicillin resistant *S. aureus*, **FA-MRSA**=Food-associated methicillin resistant *S. aureus*, **CA-MRSA**=Community-associated methicillin resistant *S. aureus*, (for the abbreviations of antibiotics, see keys of Table I)

Table 2. Prevalence, drug resistance and molecular types of LA-MRSA, FA-MRSA and CA-MRSA in various cities of Pakistan

Drug resistanceof HA-MRSA strains found in Pakistan was derived as statistical mean of resistance against each drug reported in various cities (Fig. 3). Accordingly, all strains of HA-MRSA were noticed entirely resistant to Penicillin, Methicillin and Ampicillin followed by; 90% Oxacillin-resistant, 87% Cefoxitin-resistant, 84% Amoxicillin-clavulanate-resistant, 77% Tobramycin-resistant, 71% Erythromycin and Ciprofloxacin-resistant, 67% Norfloxacin, Clarithromycin, Ofloxacin and Gentamycin-resistant, 56% Doxycycline-resistant, 54%

Tetracycline and Sulfamethoxazole-trimethoprim-resistant, 49% Sparfloxacin and Clindamycin, 47% Amikacin-resistant, 44% Fosfomycin and Meropenem-resistant, 38% Fusidic acid and Imipenem-resistant, 37% Levofloxacin-resistant, 31% Chloramphenicol-resistant, 23% Rifampicin-resistant, 14% Minocycline-resistant, 8% Teicoplanin-resistant, 7% Linezolid-resistant, 6% Tigecycline-resistant, 5% Vancomycin-resistant, and 2%Quinupristin-dalfopristin-resistant strains.

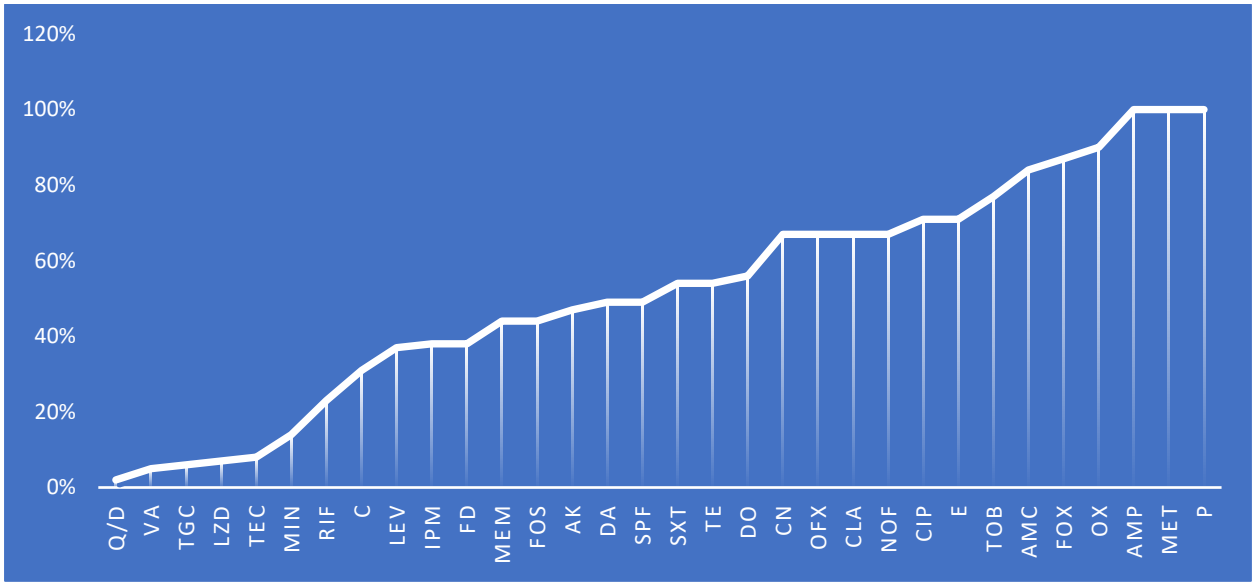


Fig 3. Drug resistance scenario of HA-MRSA in Pakistan

Interestingly, Tobramycin, Ofloxacin, Gentamicin, Sulfamethoxazole-trimethoprim, Clindamycin, and Amikacin resistance was greater in CA-MRSA strains in comparison with HA-MRSA (Fig. 4). However, Azithromycin, Doxycycline, Fusidic acid, Linezolid and Vancomycin resistance was noticed higher in HA-MRSA strains comparatively. Standard deviation was found 30.9 between the drugs resistance of HA-MRSA and CA-MRSA strains, and p-value (0.035) was calculated by two tailed t-test using the drugs resistance values of both types of MRSA.

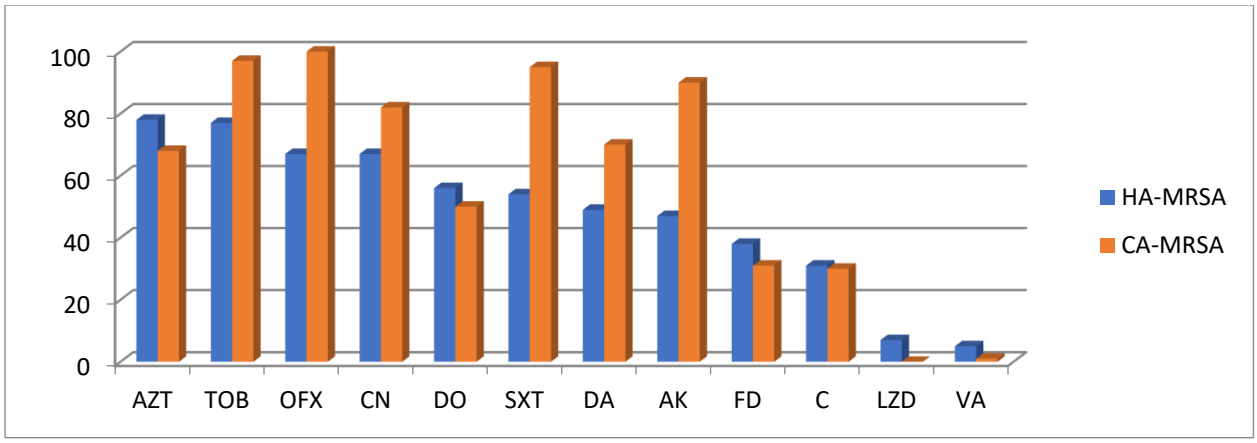


Fig 4. Drug resistance comparison between HA-MRSA and CA-MRSA

Drug resistance among HA-MRSA strains of Karachi, Lahore, Islamabad, Hyderabad, and Peshawar was analyzed (Fig. 5). Correspondingly, resistance against Tetracycline, Ciprofloxacin, Gentamycin, Erythromycin, Doxycycline, Chloramphenicol, Sulfamethoxazole-trimethoprim, Amikacin, Clindamycin, Fusidic acid, Rifampicin, Vancomycin and Linezolid was noticeably highest in HA-MRSA strains of Lahore (95%), Peshawar (88%), Islamabad (82%), Karachi (83%), Karachi (80%), Peshawar (74%), Lahore (68%), Lahore (67%), Karachi (59%), Karachi (44%), Islamabad (37%), Lahore (7%), and Hyderabad (8%), respectively. However, least resistance <10% to Linezolid and Vancomycin in all five cities was endorsed. Minimum inhibitory concentrations (MICs) of various miscellaneous antibiotics were noted in HA-MRSA strains of Pakistan (Table. 1). Increase in MICs of Vancomycin, Teicoplanin and Linezolid has been recorded in addition to other antibiotics. This situation is alarming as these antibiotics are the last resort for the treatment of HA-MRSA.

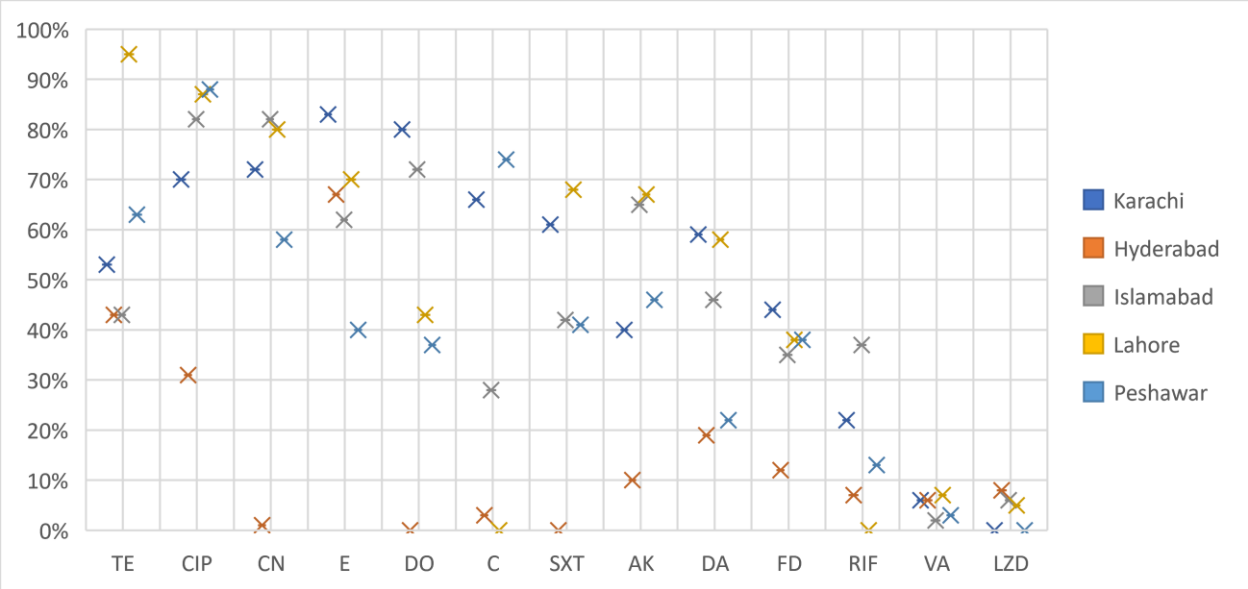
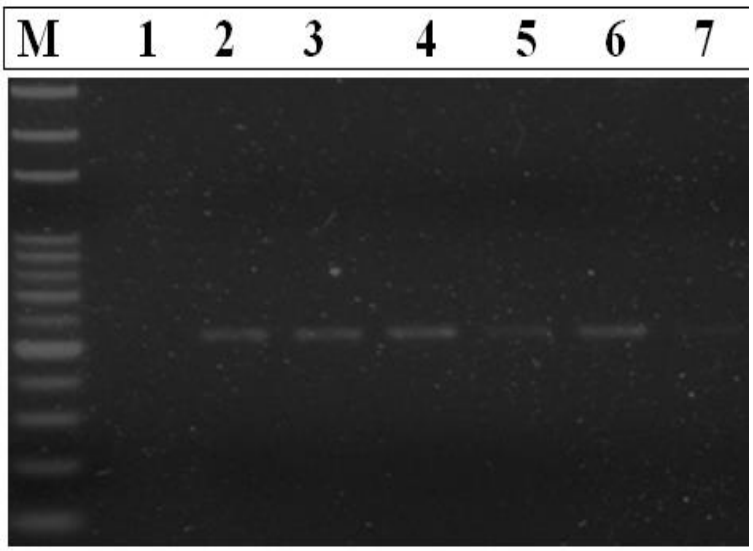


Fig 5. Drug resistance ratio of H-MRSA observed in major cities of Pakistan

Various drug resistance genes and MRSA-strain typing were recorded in Pakistan including present study(Table. 1, 2 and Fig. 6). In Faisalabad 73% and 78% of HA-MRSA were acclaimed to bear *vanA* (Vancomycin resistance gene) and *cfr* (Linezolid resistance gene), indicating the presence of VRSA and Linezolid resistant strains (Azhar et al., 2017).In Hyderabad, twenty HA-MRSA strains harbored *mecA* (Methicillin resistance gene) (Brohi and Noor, 2017). The presence of resistance factors in HA-MRSA strains (MRSA *agrI*, *agrII*, *agrIII*, *SCCmec* types; I, II, III, IV, VI) of Islamabad were witnessed to bear Trimethoprim-resistancegene*dfr(*B), tetracycline-resistance genes;*tet*(K), *tet*(M), Methicillin resistance genes; *mec*A, *mec*C, Beta-lactam or Penicillin-resistance gene *blaZ*, Quinolones-resistance genes; *gyr*A, *gyr*B, *grl*A, *grl*B, Rifampicin-resistance gene;*rop*B, Aminoglycoside-resistance gene *aacA-aph*D1, Erythromycin or Clindamycin-resistance genes; *erm*(A), *erm*(C), Fosfomycin-resistance gene *fos*(A), and

Streptogramin-resistance genes; *vat*(C), *vat*(B) and *vat*(A) genes at different rates indicated in Table. 1 (Khan et al., 2020). Furthermore, varied strains of HA-MRSA; *agrI*, *agrII*, *agrIII*, CC8, CC30, *SCCmec* types; III, IIIa, and IV, and *ST239*, *ST8* and *ST113*with distinct ratios were observed in Islamabad (Table. 1) (Shabir et al., 2010; Khan et al., 2014).



Keys: M=100bp ladder, 1-7= PCR products of *mecA* gene

Fig 6. Amplified product of *mecA* gene as shown by bands of 533bp (present study)

In Karachi, *mec*A gene was found in 100% of HA-MRSA (Taj et al., 2010). Similarly, present study marked the presence of *mecA* gene in 96% of HA-MRSA (Table.1, Fig. 6). In Lahore, *mec*A, and *van*A genes were reported in 33 to 100% and 30% of HA-MRSA, respectively (Iqbal et al., 2018; Saeed et al., 2020). Further, virulence genes like *spa*1, *spa*2, *spa*3, *spa*4, and *luk*S/F-PV were also detected in the HA-MRSA strains of Lahore (Iqbal et al., 2018; Tariq and Javed, 2019). HA-MRSA strains; *SCCmec* IV, *SCCmec* V, ST772-MRSA-V (PVL+), Bengal Bay clone, CC6-MRSA-IV, WA MRSA-51, CC8-MRSA-IV (PVL+/ACME+), USA300, CC509-MRSA-IV with varied percentage have been detected in Malakand. Moreover, *mec*A, *blaZ*, Lincosamide-resistance gene *lin*A, Macrolide-resistance genes; *mpb*BM, *msr*A, Aminoglycoside resistance genes; *aacA-aphD*, *aal*D, *aph*A3, Streptothricin-resistance gene *sat*, Tetracycline-resistance gene *tet*K were also disclosed in such strains (Madzgalla et al., 2016). In Peshawar, many pulsotypes of HA-MRSA; C1aaba, C1a4b, C1b, C2a, C1b, C2a, C1a5, D, E1aaa, E1aab, G1, H, and I1 have been recognized(Table. 1) (Ahmad et al., 2014).

In Faisalabad, 34% of LA-MRSA have been found to possess *mecA* gene (Aqib et al., 2017). Miscellaneous molecular types of LA-MRSA (ST8-t8645-MRSA-IV, USA300, ST772-t657-MRSA-IV, ST772-t8645-MRSA-IV, Bengal Bay community-associated MRSA clone) have been encountered in Haripur. Moreover, such molecular types have been noticed to harbor *mec*A, *aac-aph*, and *pvl* (Panton valentine leucocidin) gene (Syed et al., 2017). Interestingly in Islamabad, FA-MRSA bearing *mecA*, and virulence genes; *coa* and *spa* have been disclosed. CA-MRSA of

Islamabad have been characterized at molecular level as pcv and *SCCmec* IV/V positive types (Sohail and Latif, 2018). FA-MRSA of Karachi were typified as *SCCmecA* IV (74%), *SCCmecA* type II (20%), and *SCCmecA* type III (5.8%) (Table. 1) (Merani et al., 2017).

Conclusions

The highest prevalence ratio (76%) of HA-MRSA was noticed in Lahore followed by (71%) and in Karachi and 70% in Islamabad, respectively. Interestingly, alarming ratio of LA-MRSA (87%) was found in Haripur. All strains of HA-MRSA were observed entirely resistant to Penicillin, Methicillin and Ampicillin followed by; 90% Oxacillin-resistant, 87% Cefoxitin-resistant, 84% Amoxicillin-clavulanate-resistant, 77% Tobramycin-resistant, 71% Erythromycin and Ciprofloxacin-resistant, and 67% Norfloxacin. Comparatively, Tobramycin, Ofloxacin, Gentamicin, Sulfamethoxazole-trimethoprim, Clindamycin, and Amikacin resistance was found greater in CA-MRSA strains. Increased MICs of Vancomycin, Teicoplanin and Linezolid among HA-MRSA strains has beenwitnessed.In Faisalabad most of HA-MRSA were acclaimed to bear *vanA* and *cfr*genesindicating the presence of VRSA and Linezolid resistant strains. Many resistance genes and molecular types (*mec*A, *dfr(*B),*mec*C,*blaZ*, *gyr*A, *gyr*B, *grl*A, *grl*B, *rop*B, *aacA-aph*D1, *erm*(A), *erm*(C), *fos*(A), *vat*(C), *vat*(B), *tet*(K), *tet*(M), *agrI*, *agrII*, *agrIII*, and *SCCmec* types; I, II, III, IV, and VI) have been detected in various isolates of HA-MRSA in Pakistan. Present study also confirmed the presence of *mecA* gene in 96% of HA-MRSA. Among LA-MRSA and FA-MRSA, appearance of molecular typesST8-t8645-MRSA-IV, USA300, ST772-t657-MRSA-IV, ST772-t8645-MRSA-IV, Bengal Bay community-associated MRSA clone, and*SCCmecA* IV, *SCCmecA* type II, and *SCCmecA* type III have been observed respectively, in Pakistan.

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