Antimicrobial resistance and molecular epidemiology of *Staphylococcus aureus* from Ulaanbaatar, Mongolia

This study aimed to characterize *Staphylococcus aureus* (*S. aureus*) strains isolated from human infections in Mongolia. Infection samples were collected at two time periods (2007-08 and 2011) by the National Center for Communicable Diseases (NCCD) in Ulaanbaatar, Mongolia. *S. aureus* isolates were characterized using polymerase chain reaction (PCR) for *mecA*, PVL, and *sasX* genes and tested for *agr* functionality. All isolates were also *spa* typed. A subset of isolates representing frequent *spa* types was subjected to antimicrobial susceptibility testing and multilocus sequence typing. Among 251 *S. aureus* isolates, genotyping demonstrated methicillin resistance in 8.8% of isolates (22/251). Approximately 28% of the tested *S. aureus* isolates were observed to be multidrug resistant (MDR). Sequence type (ST) 154 (*spa* t667) was observed to be a strain with high virulence potential, as all isolates for this spa type were positive for PVL, and 7/9 were MDR. *S. aureus* isolates of ST239 (*spa* t037) was observed to cause infections and mostly (80%) exhibited *agr* dysfunction with a high multidrug resistance profile. Additionally, a new multilocus sequence type ST2600 and new *spa* types (t10358, t10064, and t10066) were identified, warranting continued surveillance for *S. aureus* in this region.

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Introduction

In the past fifty years, *Staphylococcus aureus* (*S. aureus*) has established itself as one of the most frequent antibiotic resistant bacterial pathogens in hospitals and communities (Boucher & Corey 2008). *S. aureus* typically causes skin and soft tissue infections, but can also cause invasive infections such as bacteremia, sepsis, endocarditis, pneumonia, osteomyelitis, etc. (Hidron et al. 2008; Liu et al. 2011). In earlier years, *S. aureus* infections were commonly observed in individuals with a history of exposure to hospitals (David & Daum 2010). There has been a major epidemiologic transition since the mid-1990s when *S. aureus* was observed to cause infections in population with no known risk exposures (Bancroft 2007). The emergence of community-associated *S. aureus* has further magnified the challenge of *S. aureus* prevention and treatment practices (David & Daum 2010). Methicillin-resistant *S. aureus* (MRSA) is associated with the rise in attributable mortality due to staphylococcal infections (Cooper et al. 2004). Surveillance studies have observed a considerable difference in proportions of MRSA invasive infections in Europe ranging from < 1% in Denmark and the Netherlands to 44% in the United Kingdom and Greece (Cooper et al. 2004; Kock et al. 2010). Nationwide surveillance for invasive MRSA infections conducted in the United States reported about 94,000 cases resulting in approximately 18,000 deaths (Klevens et al. 2007). Worldwide, rates of MRSA have been increasing as observed from data obtained via surveillance initiatives by the National Nosocomial Surveillance System (NNIS) and the European Antimicrobial Resistance Surveillance System (EARSS) (Fridkin et al. 2002; Grundmann et al. 2006; Tiemersma et al. 2004; Turnidge & Bell 2000). Nevertheless, a major concern is the lack of data in many countries, particularly the developing countries, as this could potentially result in global transmission of undetected MRSA strains (Azeez-Akande 2010; Molton et al. 2013).

Mongolia is a relatively small country in North-East Asia locked for the most part between China in the south and Russia in the north with a population of approximately 2.8 million (Bataar et al. 2010; Ider et al. 2010; Mongolia 2013). The capital city of Ulaanbaatar is home to roughly half of the country's population (Bank 2011). Infectious diseases still figure in the top 10 causes of death in the country, with sepsis being a common diagnosis among ICU patients (Bataar et al. 2010). The evolving political and economic changes in Mongolia have impacted the working of laboratory networks, data collection and management systems, and training of healthcare professionals in identification and prevention of hospital-acquired infections (Ider et al. 2010). There has also been a disruption of funds to hospitals to conduct surveillance for multidrug-resistant organisms. Due to insufficient laboratory capacity, hospitals in Mongolia use culture testing methods only when empiric therapy fails (Mongolia 2007). This process could potentially propagate antimicrobial resistance in pathogens such as *S. aureus*. In addition, a large herder population in Mongolia with a livestock population of 43 million animals increases the risk of transmission of zoonotic infections (Mongolia 2010).

A community-based survey conducted by the World Health Organization (WHO) in Ulaanbaatar between March and April 2009 observed a prevalence of 42% in the use of non-prescription antibiotics among children less than 5 years of age. This proportion is much higher than other regions such as rural communities in Vietnam (12%) and a Chinese city (36%) (Togoobaatar et al. 2010). The study found approximately 50% of the children in participating households were prescribed antibiotics, of which roughly 51% children were given both prescribed and non-prescribed antibiotics by their caregiver (Togoobaatar et al. 2010). In a developing country such as Mongolia, unconditional use of antibiotics is of particular concern (Stefani & Goglio 2010). Selective pressures such as the unrestricted use of antibiotics and inadequate compliance to antibiotic regime in conjunction with inadequate surveillance for antimicrobial resistance are

some of the important reasons for the emergence of highly resistant *S. aureus* strains (Grundmann et al. 2006; Stefani & Goglio 2010).

High population density, urbanization, inadequate infection control policies, exploding antibiotic use, and lack of appropriate healthcare delivery are some of the established social risk factors for colonization and transmission of *S. aureus* strains in hospitals and communities (Charlebois et al. 2002; Chen et al. 2011; Clements et al. 2008; Henderson 2006; Rehm & Tice 2010). There are very few studies in the published literature on the epidemiology of *S. aureus* in Mongolia. A study conducted in 2006 in Ulaanbaatar analyzed *S. aureus* infection isolates obtained from four university hospitals (Orth et al. 2006). Analysis using molecular methods and antibiotic susceptibility testing in isolates from this study determined the prevalence of MRSA to be very low (2.9%) (Orth et al. 2006). However, this study only included isolates collected between 2000 and 2002 and characterized only the six MRSA isolates identified in their cohort of *S. aureus* isolates. The aim of our study is to bridge the gap in *S. aureus* literature from Mongolia, and determine the *S. aureus* molecular epidemiology and antimicrobial resistance patterns in Mongolia.

Materials and Methods

This is an observational study conducted during two time periods (2007-08 and 2011) to investigate the prevalence of MRSA infections, and to characterize the *S. aureus* strains causing these infections in Mongolia. The University of Iowa IRB evaluated this project and determined that it did not qualify as human subjects research. To accomplish the study objective, we collaborated with the National Center for Communicable Diseases (NCCD) in Ulaanbaatar. The NCCD has an established Hospital Related Infection Surveillance and Research Unit (HRISRU)

(Ider et al. 2010). This study characterized *S. aureus* isolated from human infections in a convenience sample obtained from the NCCD.

S. aureus samples collected from various microbiology laboratories in 2011 was compared to banked *S. aureus* isolates in the period 2007-08. Patient information such as age, gender, and sample type were available only for the 2007-08 infection samples. Isolates were labeled "wound" if collected from surgical site infections or wound samples. All urine samples collected in 2007-08 were obtained from voided urine i.e. none of these patients were catheterized.

Biochemical testing and DNA isolation

Isolates were grown and confirmed to be *S. aureus* as described previously (O'Brien et al. 2012). All *S. aureus* isolates were frozen and stored in glycerol broth solution at -80^{0}C for future use. *S. aureus* DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison WI) following manufacturer's instructions.

Antimicrobial Susceptibility Testing (AST)

The antimicrobial susceptibility of isolates were tested by the broth microdilution method in accordance with the Clinical Laboratory Standards Institute (CLSI) standards (CLSI 2012). Isolates were tested for susceptibility to the following 11 antimicrobials: oxacillin, gentamicin, erythromycin, clindamycin, tetracycline, trimethoprim/sulfamethaxozole (TMP/SMX), imipenam, levofloxacin, linezolid, vancomycin, and daptomycin. Resistance to high-level mupirocin and inducible clindamycin resistance (ICR) were also examined. All AST-confirmed MRSA isolates and MSSA isolates non-susceptible to ≥ 1 antibiotic in ≥3 discrete antimicrobial categories were classified as multidrug resistant (MDR), as per a recently published report on standardization of bacterial antimicrobial resistance profiles (Magiorakos et al. 2012).

S. aureus genetic analysis

Amplification of the *spa* fragment was performed using methods and primers, as described previously (Shopsin et al. 1999). Identification of *spa* type for each isolate and the Based Upon Repeat Pattern (BURP) analysis to identify *spa* cluster complexes (*spa*CCs) was performed using the Ridom StaphType software (version2.2.1; Ridom GmbH, Würzburg, Germany) (Harmsen et al. 2003; Mellmann et al. 2008; Mellmann et al. 2007; Strommenger et al. 2008). The allelic profile of *S. aureus* isolates was determined using the Multilocus sequence typing (MLST) (Enright et al. 2000). All isolates were tested for the Panton-Valentine leukocidin (PVL) (*luk*S-PV and *luk*F-PV) (Lina et al. 1999), *mecA* (Bosgelmez-Tinaz et al. 2006), and the *sasX* gene as previously described (Holden et al. 2010; Li et al. 2012). Studies observed that presence of the *sasX* gene in *S. aureus* potentially increased its virulence capacity by boosting the bacterial defense mechanism (Li et al. 2012). These studies observed the *sasX* gene in 95% and 72% of ST239 isolates in the year 2003-05 and 2009-11, respectively (Holden et al. 2010; Li et al. 2012). Identified positive and negative controls were used in all molecular assays.

Accessory Gene Regulator (*agr*) testing

agr functionality (functional or dysfunctional) was measured using the level of δ-hemolysin production, as described previously (Sakoulas et al. 2002; Schweizer et al. 2011; Traber & Novick 2006).

Statistical analysis

Data analysis was performed using the SAS statistical software (Version 9.3, SAS Institute Inc., Cary, NC). We used the 2-tailed Fisher's exact test, and the Wilcoxon signed-rank test to analyze categorical and continuous variables, respectively. P values ≤ 0.05 were considered statistically significant for associations between explanatory variables such as age, gender, and type of infection and *S. aureus spa* type, *mecA*, PVL and *agr* functionality. Antimicrobial susceptibility results were analyzed by year of data collection to observe trends in resistance for each tested

antibiotic. Association between *S. aureus* MDR, and *mecA*, PVL, *agr* functionality, and *spa* types were also assessed.

Results

Patient and sample characteristics

In total we analyzed 252 potential *S. aureus* isolates, 198 from 2011 and 54 from 2007-08 isolate collections. Of these, 251 were confirmed to be *S. aureus* isolates. Patient demographics and sample characteristics were available only for the 53 isolates collected in 2007-08. The age of enrolled patients ranged from 1day- 82 years (median: 24 years). Of the 53 patients, 31 (58.5%) were females and 22 (41.5%) males. Approximately 43% of the *S. aureus* were isolated from wound samples.

Molecular typing

We observed a high prevalence of the *mecA* gene in isolates from the 2007-08 collection (**Table 1**). All isolates tested negative for the *sasX* gene. The cohort of *S. aureus* isolates from 2007-08 had a significantly higher PVL prevalence (85%) with a significant difference between the two time periods (p=3.496E-12). There was a moderate statistically significant difference in *agr* function (p=0.023) with greater proportion of functional isolates in the 2011 isolate collection (**Table 1**). A borderline significance was observed between age and PVL positivity in that most of our PVL-positive *S. aureus* isolates were obtained from older individuals (p=0.045, other data not shown). A moderately significant association was observed between presence of *mecA* and *agr* functionality (p=0.033).

Antimicrobial susceptibility patterns

A subset of *S. aureus* isolates (80/251, ~32%) were tested for antimicrobial susceptibility, based on the frequency of *spa* types. The proportion of *S. aureus* isolates that were MRSA (MDR) was greater in 2011 (**Fig 1**). Interestingly, we did not observe any *S. aureus* isolates that belonged to

the MSSA-MDR category in 2011. Isolates in both years have comparable proportions of MSSA that do not meet the MDR criteria. There was no significant difference in the proportion of MDR isolates between the two study periods (p=0.092).

There was a visible increase in the proportion of isolates resistant to oxacillin, tetracycline and gentamicin between the two time periods (**Fig 1**). Resistance to TMP/SMX appears to have decreased during the study periods. We also observed a wider spectrum of resistance in the 2011 isolates as there is additional resistance to clindamycin, imipenam, and ICR. We did not identify high-level mupirocin resistance in our isolates. Overall, the prevalence of antibiotic resistance in tested *S. aureus* isolates was 38.8% (71% vs. 29%).We observed good concordance in *mecA* positivity and phenotypic expression of oxacillin resistance among tested *S. aureus* isolates. Three isolates were observed to have discordant oxacillin-resistance phenotype-genotype (data not shown).

A borderline significant association was observed between antimicrobial resistance in tested *S. aureus* isolates and *agr* functionality (p=0.0488). Of the 22 MDR isolates, roughly 55% (12/22) were positive for the PVL gene, about 86% (19/22) positive for the *mecA* gene, and approximately 64% (14/22) had a functional *agr* phenotype.

spa type distribution (BURP) and Multilocus sequence type (MLST)

Eleven confirmed *S. aureus* isolates were identified as “non-typeable” after at least two attempts to sequence the *spa* gene. We identified 63 distinct *spa* types in our study (**Table 2**). The most common *spa* types in 2007-08 were t589 (13%), t3465 (13%), and t435 (11%). *spa* types t435 (10%), t589 (8%), t5288 (7.5%), and 7% each t1460 and t8677 were the most frequently occurring strains in 2011.

BURP analysis revealed clustering around founder *spa* types t589 (2011), t435 (2007-08 and 2011), t3465 (2007-08), and t8677 (2011) (**Fig 2**). We observed greater genetic diversity among the MSSA isolates and identified three new *spa* types (t10064, t10066, and t10358) in the 2011 collection. *spa* cluster complex 667 (*spa*CC 667) constituted only 5% of all the strains. Nevertheless, isolates in this group had high PVL prevalence (100%), high *mecA* prevalence (72.7%), 100% functional *agr* isolates, and high multi-drug resistance. *spa*CC 037 also had high multidrug resistance but had lower presence of PVL (10%), *mecA* (50%) and functional *agr* (60%). Singleton *spa* types were t002, t126, t156, t521, t647, t803, t1451, t3329 and t8039. None of the tested singletons appeared to be MDR.

A subset of *S. aureus* isolates from the most frequent *spa* types in each time period were tested by MLST revealing the following information: t021 (ST30), t037 (ST239), t084 (ST15), t435 (ST121) and t667 (ST154). *spa* types t1460, t5288, and t589 were observed to be ST45 in our study. One t589 isolate from the 2011 collection was identified to be a new sequence type ST2600.

Discussion

The prevalence of MRSA among clinical *S. aureus* isolates obtained from Mongolia was 8.8%. A study conducted in 2006 in Mongolia analyzing *S. aureus* isolates collected between 2000-02 found a low prevalence of MRSA (2.9%) by susceptibility testing (Orth et al. 2006). Our study is based on a convenience sample of Mongolian *S. aureus* isolates. Nevertheless, we could infer with caution that there may be an increase in the prevalence of MRSA in 2011 reflected by the absence of MDR-MSSA isolates. These observations suggest that infections due to MRSA may be increasing in Mongolia, potentially replacing the MSSA strains while gaining resistance to a wide range of antibiotics. Due to lack of published *S. aureus* data from Mongolia we compared our results to studies from China and Russia since there is a potential for transmission given its

geographical proximity. China reported a mean rate of 50.4% for MRSA prevalence in 2005 with considerable variations even within the country (Chu et al. 2013; Song et al. 2013; Wang et al. 2008; Yu et al. 2012; Zhao et al. 2012). The proportion of methicillin resistance reported from *S. aureus* in Russia varied from 18% (Vorobieva et al. 2008) to 48% (Baranovich et al. 2010). Our study data observed a lower prevalence of MRSA relative to the neighboring countries. Nevertheless, given the convenience sample this may be an underestimate of the 'true' prevalence of MRSA in Mongolia.

Our study observed greater genetic diversity in the MSSA isolates compared to the MRSA isolates. This is consistent with the finding that MRSA could potentially emerge from existing MSSA clones by acquisition of the SCC*mec* complex (Hanssen & Ericson Sollid 2006; Song et al. 2013). Hence it is crucial to implement surveillance protocols for *S. aureus* particularly in developing countries such as Mongolia that has myriad factors contributing to antimicrobial resistance. We observed a significant association between antimicrobial resistance and functionality of the *agr* system suggesting a potential influence of antimicrobial resistance on the fitness of the pathogen via the *agr* or vice versa (Paulander et al. 2013). In addition, there appeared to be an association between methicillin resistance and *agr* functionality consistent with previous findings on the regulation of drug resistance in MRSA (Hao et al. 2012). These findings could potentially influence treatment options for *S. aureus* infections in Mongolia by considering the trade-off between fitness of the strain and its range of antimicrobial resistance.

Sequence types identified by MLST were consistent across time, regardless of the combinations of *mecA*, PVL and *agr* functionality suggesting that there may be only minimal or absent mutations in the *S. aureus* core genome. Our data suggests that the MRSA clone ST239-*spa* t037 is being transmitted amongst the population. This MLST type was also reported in the previous study from Mongolia, suggesting that ST239 could potentially be the dominant MRSA clone

circulating in the country (Orth et al. 2006). ST239, a *S. aureus* bacterial hybrid formed by the admixture of MRSA clonal complexes ST30 and ST8 has been reported to be the dominant hospital clone in Asia (Aires de Sousa et al. 2003; Baranovich et al. 2010; Song et al. 2013; Xu et al. 2009; Yamamoto et al. 2012; Yu et al. 2012), Europe (Alp et al. 2009; Szczepanik et al. 2007; Wisplinghoff et al. 2005), South America (Carvalho et al. 2010; Vivoni et al. 2006), and the Middle East (Cirlan et al. 2005) and even responsible for an outbreak of device-associated bacteremia in Europe (Edgeworth et al. 2007). In accordance with previous reports, all three identified ST239 *S. aureus* strains in our study were observed to be MDR (Smyth et al. 2010). The proportion of multidrug resistance was observed to be higher in 2011 relative to 2007-08, albeit this difference was not statistically significant. Reports on *S. aureus* multidrug resistance observed variable rates ranging from ~29% - 100% from China (Chao et al. 2013; Chen et al. 2009; Wang et al. 2012) and about 90% Russian MRSA isolates (Baranovich et al. 2010). Our results suggest that there may be a surge in *S. aureus* antimicrobial resistance in the endogenous strains in Mongolia, potentially triggered by unrestricted use of antibiotics.

None of our isolates, including the identified ST239 isolates, exhibited the presence of the *sasX* gene, suggesting its prevalence may be low or absent in Mongolia. We did not identify any livestock-associated strains in our isolate collection, although several reports from China have observed the presence of these strains in human and animal population (Zhao et al. 2012).

Our study has several limitations. *S. aureus* isolates were collected as a convenience sample and could not be consistently linked to important patient information, particularly the 2011 collection that had a greater sample size for *S. aureus* isolates. We were also unable to identify any duplicate isolates. In addition, isolates from both time periods were not collected in a systematic manner adding to the selection bias. Hence, results from this study may reflect only a snapshot of the 'true' estimate of *S. aureus* infections in Mongolia. Conclusions drawn from this study could be

used as preliminary results to develop further studies with a stronger prospective study design. Nevertheless, there are not many studies from Mongolia and our study adds valuable information on the molecular epidemiology of *S. aureus* infections in Mongolia.

Another drawback of our study was the inability to differentiate the potential origin of *S. aureus* strains as healthcare associated (HA-) versus community associated (CA-) since we did not have access to the date of admission before *S. aureus* isolation from the infection. Given that in recent times there has been a gradual blurring in the origin of *S. aureus* strains the reliability of this differentiation may be questionable (Mera et al. 2011). In addition, we also did not test all *S. aureus* isolates in our collection for antimicrobial susceptibility.

Conclusion

In summary, our study observed an increasing prevalence of MRSA by AST, and recorded an antimicrobial resistance rate of 38.8% and a multidrug resistance rate of 28% in *S. aureus* isolates from Mongolia. We also observed the presence of previously identified *S. aureus* strains such as ST239 and ST30 adding its virulence potential to an existing burden of antimicrobial resistance. Regular surveillance and implementation of stricter policies for antimicrobial use is warranted to prevent further transmission of *S. aureus* in Mongolia.

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Table 1(on next page)

Prevalence of *S. aureus* genes

Gene tested	Frequency (%) 2007-08 (N=53)	2011(N=198)	p-value
mecA			
Positive	6 (11.3)	16 (8.1)	0.459
Negative	47 (88.7)	182 (91.9)	
PVL			
Positive	45 (84.9)	63 (31.8)	<0.001
Negative	8 (15.1)	135 (68.2)	
agr*			
Functional	39 (84.8)	188 (95.0)	0.015
Dysfunctional	7 (15.2)	10 (5.1)	

*Seven *S. aureus* isolates from 2007-08 did not grow for *agr* testing, significant if p≤0.05

Table 2(on next page)

Distribution of *spa* types and *spa*CC among Mongolia *S. aureus* isolates

***spa*CC type**	**Study assigned *spa*CC**	**No. (%) of strains**	***spa* types***
*spa*CC 589	CC1	78 (37.9)	**t5288**, t3126, t1460, t589, t4153, t6242, t2397, t3103, t630, t073, t3219, t7043, t102, t550, t722, t908, t10064
*spa*CC 435	CC2	62 (30.1)	**t435**, t270, t2392, t169, t272, t284, t159, t308, t8762, t6870, t2087, t1441
*spa*CC 8677	CC3	34 (16.5)	**t8677**, t4049, t3156, t10066, t4473, t3465
*spa*CC 037	CC4	10 (4.9)	**t037**, t021, t074
*spa*CC 084	CC5	4 (1.9)	**t084**, t085, t1038
*spa*CC 1194	CC6	3 (1.5)	**t1194**, t1710
*spa*CC 667	CC7	11 (5.3)	**t667**, t2832
*spa*CC 1010	CC8	2 (1.0)	**t1010**, t081
*spa*CC 8	CC9	2 (1.0)	**t008**, t024
Total		**206**	

*Bolded *spa* type is the founder for that *spa*CC
BURP clusters formed of 240 *S. aureus* isolates
25 singleton and 11 non-typeable isolates excluded from BURP clusters

Figure 1

Antimicrobial resistance and MDR trends in Mongolia *S. aureus* isolates

*TMP/SMX = trimethoprim/sulfamethaxozole, MDR = multi-drug resistant, MSSA = methicillin-susceptible *S. aureus*, ICR = Inducible Clindamycin Resistance

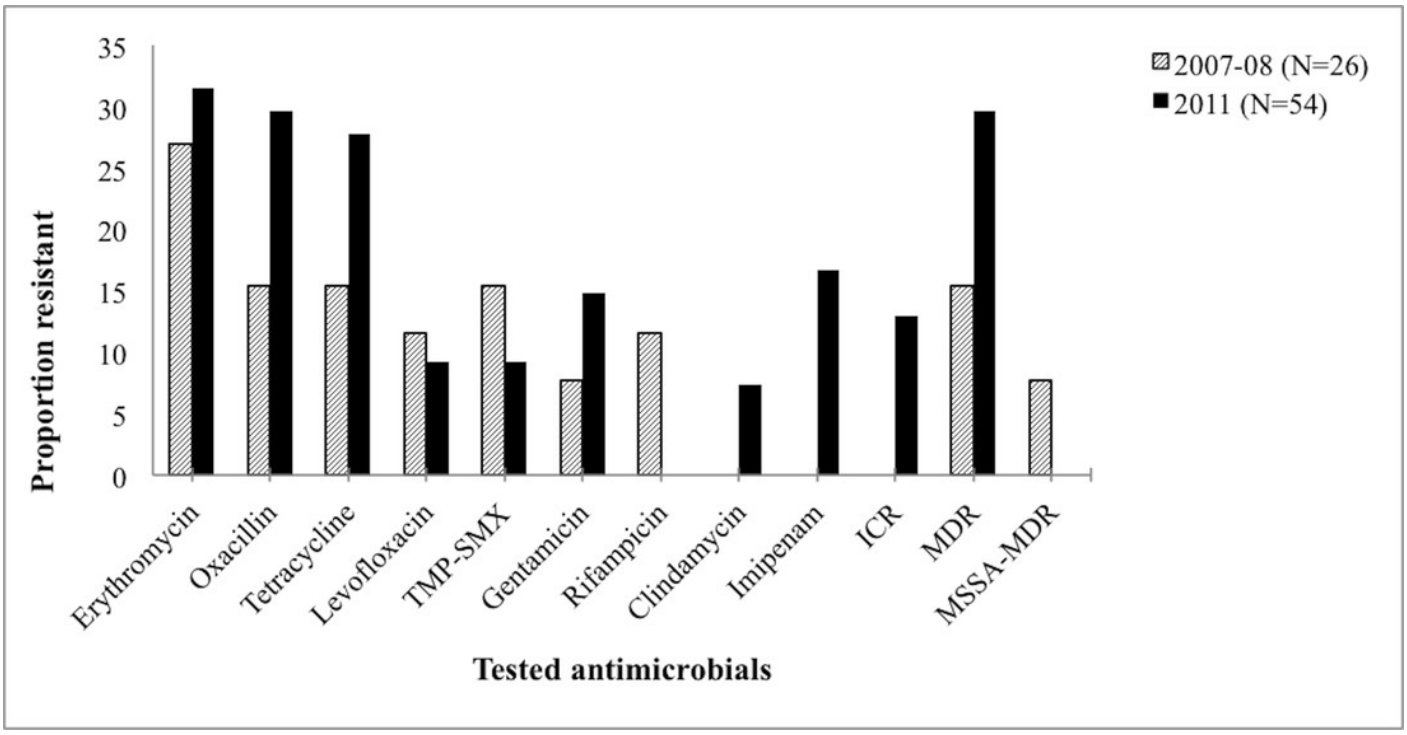

Figure 2

Population snapshot for Based-Upon Repeat Pattern (BURP) analysis

BURP grouping using default parameters resulted in 8 *spa*CCs and excluded 5 *spa* types (t026, t132, t517, t2493, and t10358). Each dot represents a unique *spa* type. Diameter of a dot is proportional to the quantity of corresponding *spa* type. Blue dots = group founders (i.e. *spa* type with the highest score within the CC). Yellow dots = subfounders with second highest score. If two or more *spa* types have the same highest founder score they are illustrated in blue. The distance between linked and/or unlinked *spa* types do not concern the genetic distance between them.