



SJMLS-3(3)-005

Effects of Storage on Resistant *Staphylococcus Aureus* Strains

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Abstract

Storage of bacterial isolates is critical for research, diagnostic, and teaching purposes. The various methods of storage depend on intended use of the organism and time, which ranges from short-term to long-term storage. Other methods include Cryoprotectants, Freezing samples, Freeze-drying. Storage effects on bacteria may have important implications, in which Methicillin Resistant *Staphylococcus aureus* (MRSA) strains are not exceptional. A literature search was carried out and identified 84 papers. After exclusions, a total of 28 were found relevant. This review explored the fact that, effects of storage on MRSA strains are enormous. This include reduced susceptibility to antibiotics, loss of *mecA* gene, reduced or loss of viability among others. It is therefore of paramount importance that more concern should be given to the influence that storage conditions have on characteristics of the stored strains of resistant *Staphylococcus* species. In order to determine the effects of storage on resistant *Staphylococcus aureus*, various relevant literatures using internet, textbooks, journals, periodicals *e. t. c.* were explored and information relevant to the subject matter were obtained. Recommendations on possible ways for proper maintenance and preservation of resistant *Staphylococcus aureus* strains were made.

Keywords: Resistance, *Staphylococcus aureus*, genes, storage, effect.

Introduction

Resistant *Staphylococcus aureus* refers to a

group of gram-positive bacteria that are genetically different from other strains of *Staphylococcus aureus*. Methicillin resistant *Staphylococcus aureus* (MRSA) is responsible for several infections that are difficult to treat in humans. MRSA is any strain of *S. aureus* that has developed, through horizontal gene transfer and natural selection, multiple drug resistance to beta-lactam antibiotics. β -lactam antibiotics are a broad spectrum group which includes some penams – penicillin derivatives such as methicillin and oxacillin, and the Cephalosporins (Gurusamy *et al.*, 2013). Other strains of resistant *S. aureus* have emerged that are resistant to oxacillin, clindamycin, teicoplanin, and erythromycin. These resistant strains may or may not possess the *mecA* gene. *S. aureus* has also developed resistance to vancomycin (VRSA).

Resistant *Staph. aureus* are common in hospitals, prisons, and nursing homes, where people with open wounds, invasive devices such as catheters, and weakened immune systems are at greater risk of hospital-acquired infection. The organism began as a hospital-acquired infection, but has become community-acquired as well as livestock-acquired (Wollima, 2017).

In humans, *S. aureus* is part of the normal microbiota present in the upper respiratory tract, (Schenck, 2016) and on skin and in the gut mucosa (Wollima, 2017). *S. aureus*, along with similar species that can colonize and act symbiotically but can cause disease if they begin to take over the tissues they have colonized or invade other tissues, have been called

"pathobionts" (Schenck *et al.*, 2017). After 72 hours, resistant *S. aureus* can take hold in human tissues and eventually become resistant to treatment.

Resistant *Staphylococcus aureus* has emerged over the past decade as a significant bacterial pathogen associated with healthcare-associated infection (Otter and French, 2011). For this reason, it is important that clinical microbiology laboratories archive representative isolates for historical purposes, as well as for longitudinal studies, where such isolates may be required within the short, medium and long-terms (Otter and French, 2011).

Therefore, the aim of this study to determine the effects of storage on resistant *Staph. aureus* isolates. Such storage effects will be of interest to Biomedical Scientists and Clinical Microbiologists with responsibility for their culture collections, so that they are aware of the survival dynamics of these organisms under such storage conditions.

Materials and Methods

A literature search was carried out and 84 journal articles were identified. After exclusions, only 28 were relevant.

Microbiology of Resistant *Staphylococcus aureus*.

Like all *Staphylococcus aureus* (also abbreviated SA at times), resistant *S. aureus* is a gram-positive, spherical (coccus) bacterium that is about 1 micron in diameter. It does not form spores and it is non-motile. It forms grape-like clusters or chains (Jensen and Lyon, 2009). Unlike Methicillin-susceptible *S. aureus* (MSSA), MRSA is slower growing on a variety of media and has been found to exist in mixed colonies of MSSA. The *mecA* gene, which confers the resistance to a number of antibiotics is present in MRSA and not in MSSA. In some instances, the *mecA* gene is present in MSSA but is not expressed. Polymerase chain reaction (PCR) testing is the most precise method in identifying MRSA strains. Specialized culture media have been developed to better differentiate between MSSA and MRSA and in some cases, it will identify specific strains that

are resistant to different antibiotics (Jensen and Lyon, 2009). Other strains of *S. aureus* have emerged that are resistant to oxacillin, clindamycin, teicoplanin, and erythromycin. These resistant strains may or may not possess the *mecA* gene. *S. aureus* has also developed resistance to vancomycin (VRSA). One strain is only partially susceptible to vancomycin and is called vancomycin-intermediate *S. aureus* (VISA). GISA is a strain of resistant *S. aureus* and stands for glycopeptide-intermediate *S. aureus* and is less susceptible to Vancomycin and teicoplanin. Resistance to antibiotics in *S. aureus* can be quantified. This done by determining the amount of the antibiotic in micrograms/milliliter must be used to inhibit growth. If *S. aureus* is inhibited at a concentration of vancomycin of less than or equal to 4 micrograms/milliliter, it is said to be susceptible. If a concentration of greater than 32 micrograms/milliliter is necessary to inhibit growth, it is said to be resistant (Winn, 2006).

Resistance Genes

Resistance to antimicrobial agents is genetically based; resistance is mediated by the acquisition of extra chromosomal genetic elements containing resistance genes. Examples include, transposable genetic elements, plasmids and genomic islands, which are transferred between bacteria through horizontal gene transfer (Jensen and Lyon, 2009). A defining characteristic of resistant *Staphylococcus aureus* is its ability to thrive in the presence of penicillin-like antibiotics, which normally prevent bacterial growth by inhibiting synthesis of cell wall material. This is due to a resistance gene, *mecA*, which stops β -lactam antibiotics from inactivating the enzymes (transpeptidases) critical for cell wall synthesis (Jensen and Lyon, 2009).

SCC*mec*

Staphylococcal cassette chromosome *mec* (SCC*mec*) is a genomic island of unknown origin containing the antibiotic resistance gene *mecA* (Pantosti *et al.*, 2007; Lowy, 2003). SCC*mec* contains additional genes beyond *mecA*, including the cytolysin gene *psm-mec*, which may suppress virulence in HA-acquired MRSA strains (Kaito *et al.*, 2011). In addition,

this locus encodes strain dependent gene regulatory RNA called *psm-mecRNA*. *SCCmec* also contains *ccrA* and *ccrB*; both genes encode recombinases that mediate the site-specific integration and excision of the *SCCmec* element from the *S. aureus* chromosome (Pantosti et al., 2007; Lowy, 2003). Currently, six unique *SCCmec* types ranging in size from 21–67 kb has been identified (Lowy, 2003) They are designated types I–VI and are distinguished by variation in *mecA* and *ccr* gene complexes (Jensen Lyon, 2009). Owing to the size of the *SCCmec* element and the constraints of horizontal gene transfer, a minimum of five clones are thought to be responsible for the spread of MRSA infections, with clonal complex (CC) (Lowy, 2003; Enright and Robinson, 2002) *SCCmec* is thought to have originated in the closely related *S. sciuri* species and transferred horizontally to *S. aureus* (Lencastre, 2001).

Different *SCCmec* genotypes confer different microbiological characteristics, such as different antimicrobial resistance rates (Kuo et al., 2012). Different genotypes are also associated with different types of infections. Types I–III *SCCmec* are large elements that typically contain additional resistance genes and are characteristically isolated from Hospital acquired methicillin resistant *Staph. aureus* (HA-MRSA) strains. Conversely, community acquired *Staph. aureus* (CA-MRSA) is associated with types IV and V, which are smaller and lack resistance genes other than *mecA* (Kuo et al., 2012).

MRSA is able to thrive in hospital settings with increased antibiotic resistance but decreased virulence - HA-MRSA targets immunocompromised, hospitalized hosts, thus a decrease in virulence is not maladaptive. In contrast, CA-MRSA tends to carry lower fitness cost *SCCmec* elements to offset the increased virulence and toxicity expression required to infect healthy hosts (Collins and Rudkin, 2010).

mecA

mecA is a biomarker gene responsible for resistance to methicillin and other β -lactam antibiotics. After acquisition of *mecA*, the gene must be integrated and localized in the *Staph. aureus* chromosome. *mecA* encodes penicillin-binding protein 2a (PBP2a), which differs from

other penicillin-binding proteins as its active site does not bind methicillin or other β -lactam antibiotics. As such, PBP2a can continue to catalyze the transpeptidation reaction required for peptidoglycan cross-linking, enabling cell wall synthesis in the presence of antibiotics. As a consequence of the inability of PBP2a to interact with β -lactam moieties, acquisition of *mecA* confers resistance to all β -lactam antibiotics in addition to methicillin (Lowy, 2003).

mecA is under the control of two regulatory genes, *mecI* and *mecR1*. *MecI* is usually bound to the *mecA* promoter and functions as a repressor. In the presence of a β -lactam antibiotic, *MecR1* initiates a signal transduction cascade that leads to transcriptional activation of *mecA* (Lowy, 2003). This is achieved by *MecR1*-mediated cleavage of *MecI*, which alleviates *MecI* repression (Jensen Lyon, 2009). *mecA* is further controlled by two co-repressors, *BlaI* and *BlaR1*. *blaI* and *blaR1* are homologous to *mecI* and *mecR1*, respectively, and normally function as regulators of *blaZ*, which is responsible for penicillin resistance (Lowy, 2003). The DNA sequences bound by *mecI* and *BlaI* are identical, therefore, *BlaI* can also bind the *mecA* operator to repress transcription of *mecA* (Collins and Rudkin, 2010).

mecC

Discovery of *mecC* MRSA

Epidemiological study of *Bovine mastitis* led to the isolation in 2007 of a *Staph aureus* isolate LGA 251 from a bulk of tank milk sample in South West England which was phenotypically MRSA (i. e. resistant to Oxacillin and Cefoxitin) (Garcia, 2011). At that time, this in itself was immediately significant because it represented the first detection of MRSA in UK dairy herd. However, the confirmatory tests for the *mecA* gene and PBP2a/2' were repeatedly negative (Garcia, 2011). Genome sequencing of LGA25 at the Wellcome Trust Sanger Institute revealed that the strain carried a novel *mecA* homologue, initially termed *mecA*_{LGA251} which was only 69% identical to the conventional *mecA* at the DNA level, and the encoded PBP2a/2' was 63% identical at amino acid level (Garcia 2011). The explained resistance of L251 and why it produced negative result by *mecA* Polymerase chain reaction (PCR) and PBP2a/2' slide agglutination (Garcia, 2011). A retrospective

search of isolate collections in UK and Denmark identified further 65 isolates positive for $mecA_{LGA251}$ not only from dairy cattle but also from humans including earliest known isolate a Danish blood isolate from 1975 (Garcia, 2011).

Similarly, a conventional $mecA$, is $mecA_{LGA251}$ is located within *Staphylococcus* Cassette Chromosome mec (SCC_{mec}) element inserted to 3' region of *orfX* (Ito, 2012). The (SCC_{mec}) sequence from LGA251 was submitted to the working group on classification of SCC and given a designation type XI (SCC_{mec}) in November, 2009. Later, $mecA_{LGA251}$ was renamed ***mecC*** in 2012 (Ito, 2012). The *mecC* gene is positioned on a SCC_{mec} XI element that has been recently identified in clonal complexes 49, 130, 425, 599 and 1943. Some known isolates of MRSA have been isolated from animals carrying *mecC* gene can colonise and cause disease in human and a wide range of host species. Although, *mecC* gene are currently rare and have only been reported in Europe. As at present, they present a diagnostic problem where there is reliance on *mecA* or PBP2a detection MRSA diagnosis (Kumurya, 2015).

Storage of Resistant *Staphylococcus Aureus* Strains

The specific length of time that a culture will remain viable in a given storage condition is dependent upon the resistant *Staph aureus* strain. Cell death during storage is inevitable but should be minimized as much as possible, which can make it easy for use. Bacterial cultures that are used regularly (i.e., daily/weekly) can be stored on agar plates or in stab cultures in a standard refrigerator at 4°C. If cultures will not be used for more than a few weeks, though, more long-term storage methods should be considered for maximum bacterial viability (Simione and Brown, 1991).

Short-term storage

Working resistant *Staph* species stocks can be streaked onto agar plates and stored at 4°C for daily or weekly use. Culture dishes should be wrapped with laboratory sealing film (plastic or paraffin) and stored upside down (agar side up) to reduce contamination and to keep both the culture and agar properly hydrated. Some bacterial strains can be stored for up to 1 year at

4°C in agar stab cultures, which are used for transporting samples to other research facilities. Stab cultures are prepared by first sterilizing strain-compatible agar (e.g., Lysogeny broth [LB] agar for *Escherichia coli*) and then transferring the warm liquid agar to screw-cap vials using the appropriate aseptic technique. After the agar has solidified, a single colony is picked from an actively growing culture using a sterile, straight wire. The wire with the bacteria is then plunged deep into the soft agar several times, and the vial is incubated at 37°C for 8–12 hours with the cap slightly loose. The vial is then sealed tightly and stored in the dark at 4°C (Simione, 1992).

Long-term storage

As mentioned above, the temperature at which frozen bacteria are stored affects how long they can be stored while remaining viable. Freezing and thawing cells at an appropriate rate and maintaining the frozen stocks at the proper storage temperature help to minimize damage from the freezing process. Also, the greater the cell density, the better the recovery is after thawing the cells. For most bacteria, a density of 10^7 cells/mL will result in adequate recovery if all conditions are properly maintained (Simione and Brown, 1991).

Cryoprotectants

As water in cells is converted to ice, solutes accumulate in the residual free water. This localized increase in salt concentration can denature biomolecules (Paoli, 2005). Furthermore, ice crystal formation can damage cell membranes. Additives that are mixed with the bacterial suspension before freezing lower the freezing point and protect cells during freezing to minimize the detrimental effects of increased solute concentration and ice crystal formation. The most commonly used cryoprotectants are dimethylsulfoxide (DMSO) and glycerol, which are typically used at 5–15% (v/v). Non-permeable additives used as cryopreservants, such as polysaccharides, proteins, and dextrans, adsorb to the surface of microorganisms and form a viscous layer that protects membranes, making these agents particularly useful for cryopreservation. Other commonly used additives include blood serum, ethylene glycol, methanol, skim milk, yeast extracts, and trypticase soy (Huba'lek, 2003).

Freezing samples

To prepare glycerol stocks, the glycerol is first autoclaved and allowed to cool. The appropriate volume of glycerol is added to a suspension of log-phase bacteria and vortexed to dissociate the cells and ensure even mixing of the bacteria with the glycerol. After aliquoting the suspension into cryogenic screw-cap vials, the cells are snap-frozen by immersing the tubes in either ethanol-dry ice or liquid nitrogen and then stored in freezers (-20 to -80°C) or liquid nitrogen (-150°C). Repeated thawing and refreezing of the bacterial stocks will reduce cell viability and should be avoided (Moore and Rene, 1975). When recovering strains with antibiotic selection markers, culturing them on selective media will ensure that the bacterial stocks were not contaminated (Moore and Rene, 1975).

Freeze-drying

Bacteria can be freeze-dried by suspending log-phase cells in a lyophilization medium and then freeze-drying the suspension. Not all bacteria can be successfully freeze-dried. Certain strains might not survive the process or die rapidly once freeze-dried. The best way to determine if a strain is amenable to freeze-drying is to empirically evaluate its stability post-freeze-drying while maintaining a live culture as a backup. Once freeze-dried, it is best to store the bacteria at or below 4°C (Shinohara, 2008).

Effects of Storage on Of Resistant *Staphylococcus Aureus* Strains

Reduced Susceptibility

Frozen-storage conditions had been reported to alter the organism's susceptibility results (Ludwig and Edwards, 2012). The reasons for these changes are uncertain, but the instability of resistant subpopulations, the loss of genetic determinants of reduced susceptibility, revertant mutations after passages on non-selective media, and a selective advantage for susceptible progenies have been proposed as possible causes of an overrepresentation of the susceptible populations after storage (Griethuysen *et al.*, 2005).

A study by Edwards (2012) on Effects of Frozen Storage on Detection of Intermediate Vancomycin Susceptibility and Heteroresistance in Methicillin-Resistant *Staphylococcus aureus*

blood isolates revealed that decreasing effectiveness of vancomycin in the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections has been linked to increasing vancomycin MIC within the susceptibility range (≤ 2 mg/liter) (5) (Dhand and Sakoulus, 2012).

In the study, it was further hypothesized that isolate storage may explain divergent results in investigations of susceptibility trends. A prospective repeated-measure design involved susceptibility testing of MRSA bloodstream isolates (BSI) at the time of isolation and at 3-month intervals. A 9-month follow-up period was chosen on the basis of previous data suggesting that changes in susceptibility occurred within this time (Edwards, 2012). The study found evidence that, in daptomycin- and vancomycin-susceptible blood isolates, MIC results from the test were inversely related to the duration of isolate storage prior to susceptibility testing (Edwards, 2012).

Loss of *mecA* Gene

Another study by Kumurya (2013) on loss of the *mecA* gene during storage of methicillin-resistant *Staphylococcus aureus* isolates in Northwestern Nigeria shows that the *mecA* gene was lost in 95.0% of 100 MRSA isolates after 2 years of storage at -80°C with the Micro bank system (Pro-lab Diagnostics, Austin, Texas).

Similarly, Griethuysen *et al.* (2004) conducted a similar research in Netherlands, titled 'Loss of the *mecA* Gene during Storage of Methicillin-Resistant *Staphylococcus aureus* Strains' after storing the MRSA strains at -80°C for a period of one year, it was discovered that 36 (14.4%) of the 250 isolates no longer harbored the *mecA* gene (Griethuysen *et al.*, 2004).

Reduction of Cell Viability

Repeated thawing and refreezing of the MRSA stocks during storage, reduces cell viability (Moore and Rene, 1975).

Loss of Culturability

Many clinical laboratories in the UK have traditionally used Dorset egg medium (DEM) as the storage medium of choice for the maintenance of their MRSA isolates. An overall, study by Sudo *et al.* (2016), using 100 MRSA clinical isolates from bacteriological achieve of

Northern Ireland Health and Social Care Microbiology Repository, demonstrated that medium- to long-term storage of 8 to 9 years of MRSA isolates on DEM at ambient temperature resulted insignificant loss of culturability, where culturability dropped by 78 % (Sudo *et al.*, 2016).

Conclusion

In conclusion, the above review indicated that storage has a profound effect on vital genomic information of resistant *Staphylococcus aureus*. *mecA* gene is significantly lost during medium to long-term storage which may lead to reduced susceptibility, losses in isolate culturability. Repeated thawing and refreezing of the resistant *S. aureus* stocks during storage reduces cell viability. Therefore, alternative storage mechanisms, including freeze-drying or frozen storage, should be put in place to protect the longevity of important resistant *Staph. aureus* culture collections.

Recommendations

It is therefore recommended that:

1. Alternative storage mechanisms, including freeze-drying or frozen storage, should be put in place to protect the longevity of important resistant *Staphylococcus aureus* culture collections.
2. Further research should be carried out on a more reliable methods for preservation of resistant *Staph. aureus* strains in order to sustain the phenotypic and genomic characteristics.
3. Attention should be given to the influence of storage conditions on characteristics of the stored strains such as antimicrobial susceptibility and viability of the microorganisms after a medium term or long-term storage period.

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Citation: Ajagbe, J.M., Kumurya, A.S., Rabi, M., Dogo, E.B., Ega, B., Tukur, A.D., Opalekundu, A.B., and Yusuf, A.A. Effects of Storage on Resistant *Staphylococcus Aureus* Strains. *Sokoto Journal of Medical Laboratory Science*;3(3): 45 - 51.

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