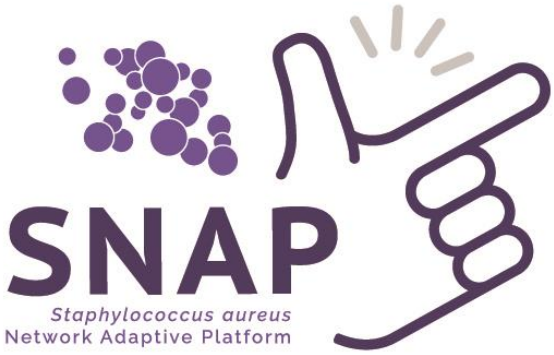

Microbiology Appendix

Staphylococcus aureus Network Adaptive Platform trial (SNAP)

Microbiology Appendix Version 2.0, dated 27 July 2023

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1. EXECUTIVE SUMMARY

- Patients are able to enter the platform following the identification and reporting of *Staphylococcus aureus* complex (includes *S. aureus, S. argenteus and S. schweitzeri*) from blood culture bottles. For the remainder of the document *Staphylococcus aureus* complex and *Staphylococcus aureus* are used interchangeably.
- Enrolment into the adjunctive antibiotic domain (i.e. clindamycin therapy) can occur immediately following identification and reporting of *Staphylococcus aureus* from blood culture bottles. Susceptibility testing, including inducible clindamycin testing, is not required for domain eligibility.
- In laboratories performing molecular testing for identification of *mecA* gene, entry into the MRSA silo of the antibiotic backbone domain can occur when Methicillin resistant *S. aureus* (MRSA) is presumptively identified by the molecular assay.
- PSSA testing is required to enrol patients in the penicillin-susceptible *S. aureus* silo of the antibiotic backbone domain.
    - The EUCAST (P1) disc is the preferred method for penicillinase testing
- Differentiation between borderline oxacillin resistant *S. aureus* (BORSA) and Methicillin resistant *S. aureus* (MRSA) is not required
- For laboratories testing for borderline oxacillin resistant *S. aureus* (BORSA), these isolates are to be considered as Methicillin resistant *S. aureus* (MRSA) with patients enrolled in the MRSA silo of the antibiotic backbone domain.
- Any oral agent used for treatment in the early oral switch domain should have tested susceptible by a validated method.

2. MICROBIOLOGY APPENDIX VERSION

The version of the Microbiology Appendix is in this document's header and on the cover page.

2.1 Version history

Version 1: Approved by the Microbiology Working Group (DSWG) on the 10th of February 2021

Version 2: Approved by the Microbiology Working Group (DSWG) on the 01 March 2023

3. MICROBIOLOGY WORKING GROUP GOVERNANCE

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4. MICROBIOLOGY WORKING GROUP AUTHORISATION

The Microbiology Working Group have read the appendix and authorise it as the official Microbiology Appendix for the SNAP trial.

Signed on behalf of the committee,

Chair  Date 01 March 2023

Prof Sebastiaan van Hal

5. INTRODUCTION

The *Staphylococcus aureus* Network Adaptive Platform (SNAP) trial will investigate treatments for *S. aureus* bacteraemia for all key antibiotic susceptibility phenotypes (penicillin-susceptible [PSSA], methicillin-susceptible, penicillin-resistant [MSSA], and methicillin-resistant [MRSA]) and in multiple management domains (including backbone antibiotic, adjunctive antibiotic, early oral switch).

The accurate and timely laboratory identification of *S. aureus* and antibiotic susceptibility phenotype is a critical component of the platform. The principal entry criteria to the platform are identification and reporting of *S. aureus* from blood cultures, and allotment to specific silos (PSSA, MSSA or MRSA) is contingent on accurate distinction of PSSA, MSSA and MRSA.

This microbiology appendix details the recommended steps for identification of *S. aureus* and determination of antibiotic susceptibility phenotype.

The platform and domain specific inclusion and exclusion criteria can be found in the relevant protocol documents. The main criteria relevant to the microbiology appendix include:

Core inclusions:

- *Staphylococcus aureus* complex grown from ≥1 blood culture
- Platform entry within 72 hours of the collection of the index blood culture

Core exclusions:

- Time of anticipated platform entry is greater than 72 hours post collection of the index blood culture
- Polymicrobial bacteraemia, defined as more than one organism (at species level) in the index blood cultures, excluding those organisms judged to be contaminants by the treating clinicians
- Known positive blood culture for *S. aureus* (of the same silo: PSSA, MSSA or MRSA) between 72 hours and 180 days prior to the time of eligibility assessment

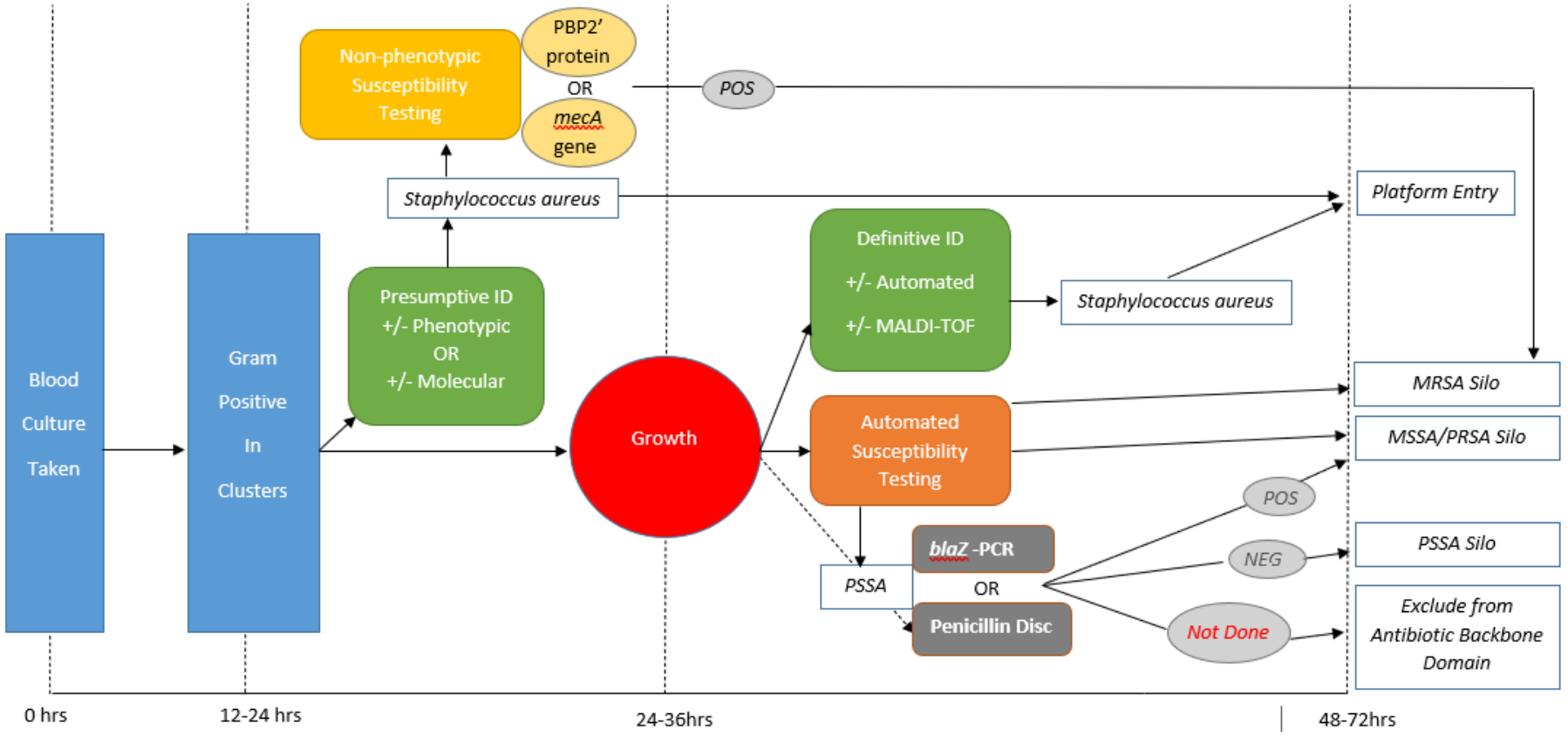
For **PSSA silo** for backbone antibiotic domain:

- Inclusion: Index blood culture isolate is penicillin-susceptible as per the Microbiology Appendix. In short, this will require phenotypic disc testing with EUCAST (a P1 disc diffusion with a feathered zone ≥26mm OR a P1 disc diffusion with zone ≥26mm and the zone edge is NOT sharp) **OR** CLSI (a P10 disc diffusion) defined criteria
- Exclusion: Patients cannot definitively be categorised as MSSA/Penicillin resistant *S. aureus* OR PSSA as described above and in the microbiological appendix.
    - Specifically in this case, if automated testing suggests PSSA (e.g., Vitek) but P1 or P10 disc susceptibility is not being performed.

For **MSSA silo** for backbone antibiotic domain:

- Inclusion: Index blood culture isolate is methicillin-susceptible but penicillin resistant as per the Microbiology Appendix
- Exclusion: Patients cannot definitively be categorised as MSSA/Penicillin resistant *S. aureus* OR PSSA as described above and in the microbiological appendix
    - Specifically in this case, if automated testing suggests PSSA (e.g., Vitek) but P1 or P10 disc susceptibility is not being performed.

6. ANTICIPATED / SUGGESTED LABORATORY WORKFLOW



7. GENERAL ANTIMICROBIAL SUSCEPTIBILITY GUIDANCE FOR SNAP

7.1. Penicillin susceptible S. aureus (PSSA) vs. methicillin susceptible S. aureus (MSSA)[1,2]

Most staphylococci are penicillinase producers. This mechanism renders them resistant to benzylpenicillin, ampicillin, amoxicillin, piperacillin and ticarcillin.

Although the use of penicillin for PSSA has advantages, guidelines tend not to recommend penicillin therapy primarily based on concerns raised about the ability of laboratories to detect penicillinase enzymes encoded by *blaZ*. The prevalence of *blaZ* in PSSA isolates (as determined by automated antimicrobial susceptibility testing) is approximately ~ 5%.

Methods of *blaZ* detection:

- Nitrocefin hydrolysis test is inadequate as it has been shown to produce false negative results
- Chromogenic cephalosporin-based beta-lactamase tests do not reliably detect staphylococcal penicillinase.
- *blaZ* PCR – not routinely done
- Penicillin disc testing: either using the EUCAST method with a P1 disc (1 μg penicillin disc) or using the CLSI method a P10 disc (10μg). Based on the data, the EUCAST method was preferred.

Penicillin	Cefoxitin	Classification	Comments
R	S	MSSA/PRSA	No additional testing required
S	S	PSSA	Penicillin disc test -> to confirm PSSA

For the purposes of SNAP:

Patients can only be enrolled into the PSSA silo of the backbone antibiotic domain if the laboratory performing susceptibility testing is able to perform confirmatory testing by either:

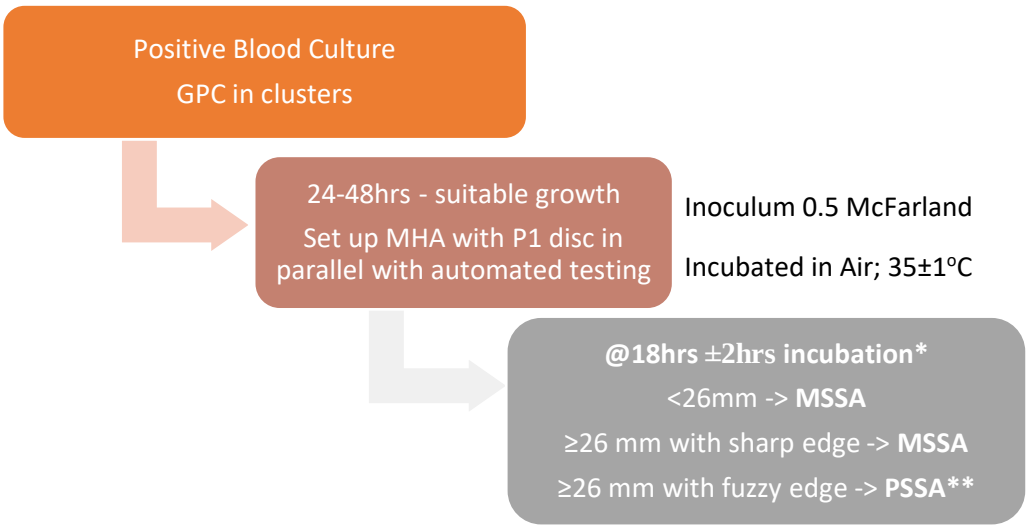
i) Excluding a penicillinase producing isolate using either the EUCAST or CLSI penicillin disc (PD).

OR

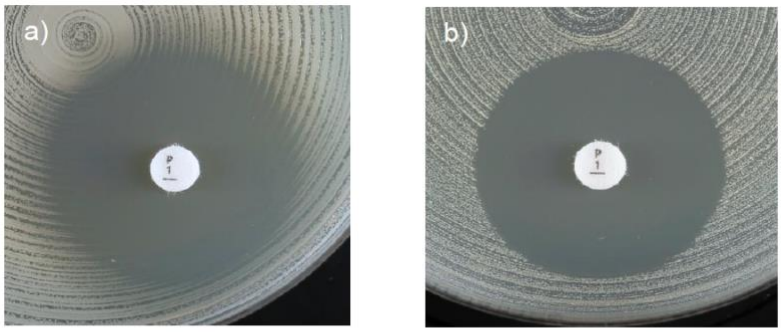
ii) Exclude the presence of the *blaZ* gene by PCR.

Please note that testing should be performed in a timely fashion to allow for enrolment within the 72-hr window and therefore it is suggested that the penicillin disc (PD) testing should be set up at the time of automated testing to allow for results to be available as soon as possible. Similarly, PCR workflows should be performed in real time.

For laboratories wishing to introduce testing, the Microbiology Working Group recommends introduction of the EUCAST method. A proposed workflow is outlined below. Although the EUCAST method is preferred over the CLSI method due to better sensitivity for *blaZ* detection, the CLSI method is an acceptable alternative.



* See illustration below



Examples of inhibition zones for *Staphylococcus aureus* with benzylpenicillin.
a) Fuzzy zone edge and zone diameter ≥ 26 mm. Report susceptible. **
b) Sharp zone edge and zone diameter ≥ 26 mm. Report resistant.

**PSSA is confirmed if the zone diameter is ≥26 mm AND the zone edge is fuzzy.

7.2. Methicillin-resistant S. aureus (MRSA) vs. borderline-oxacillin-resistant S. aureus (BORSA)[3]

Resistance to methicillin is usually associated with the presence of an alternative penicillin-binding protein, PBP2a or PBP2' encoded by chromosomal genes *mecA* or *mecC*.

Disk diffusion reliably predicts methicillin resistance. Cefoxitin disc testing is recommended by CLSI and EUCAST as it is more efficient at detecting hetero-resistant MRSA.

- EUCAST & CLSI: Cefoxitin 30µg; S≥22mm; R<22mm

S. aureus with cefoxitin MICs and oxacillin MICs values in the table below are generally methicillin resistant.

Resistance breakpoints

Method	Oxacillin MIC (mg/L)	Cefoxitin MIC (mg/L)
EUCAST	>2	>4
CLSI	≥4	≥8

Early enrolment of *Staphylococcus aureus* (i.e., before the availability of the susceptibility results) into the MRSA silo of the backbone antibiotic domain can occur provided that either:

i) The presence of the *mecA* gene is detected by PCR
OR
ii) The presence of an augmented penicillin-binding protein is detected by rapid latex slide agglutination.

Please note that testing should be performed in a timely fashion to allow for enrolment within the 72-hr window.

BORSA (borderline oxacillin resistant *S. aureus)* show borderline resistance to penicillinase-resistant penicillins (e.g. oxacillin, cloxacillin and methicillin) but do not carry a modified PBP2a.
Mechanisms include:

i) hyper-production of *blaZ*-encoded B-lactamases
ii) plasmid-mediated methicillinases or
iii) modification of alternative PBPs (usually PBP3 or PBP4). These isolates are sometimes classified as modified *S. aureus* (MODSA) to distinguish them from BORSA.
iv) mutations in core genes involved in cell-wall synthesis. Similar to VISA, these mutations have often pleiotropic phenotypes including slower growth/smaller colonies and co-resistance to cefazolin. The stronger association was found with mutations in the c-di-AMP phosphodiesterase *gdpP.* [6,7]

Prevalence among *S. aureus* isolates varies between 1.4% and 12.5% dependent on population studied. Higher rates have been detected in non-sterile sites associated with colonisation (e.g. sputum cultures in CF patients). There are no large studies examining this phenomenon in blood culture collections. The anticipated rate is <1% across all sites.

Classification of *S. aureus* isolates:

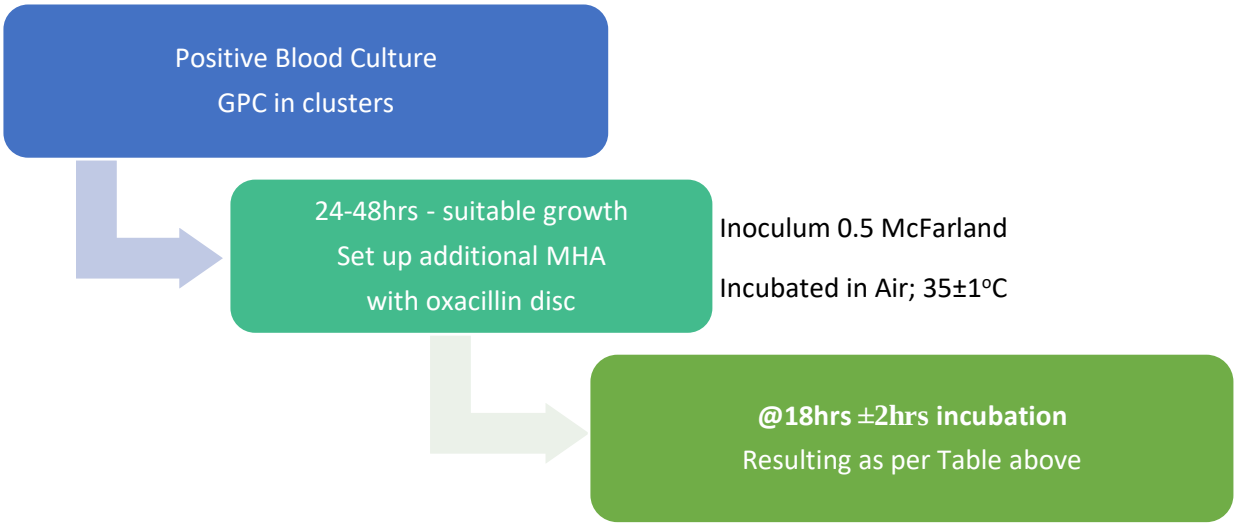
Cefoxitin	Oxacillin	mecA/mecC	Classification
R or S	R or S	present	MRSA
R	S	absent	BORSA
S	R	absent	BORSA
S	S	absent	MSSA

*See Resistance breakpoints table

For the purposes of SNAP: BORSA isolates are to be considered as MRSA.

As this phenomenon is anticipated to be small <u>additional</u> testing is **not required.**

For laboratories who wish to do further testing it is suggested that following workflow be implemented.



7.3. Inducible Clindamycin resistance

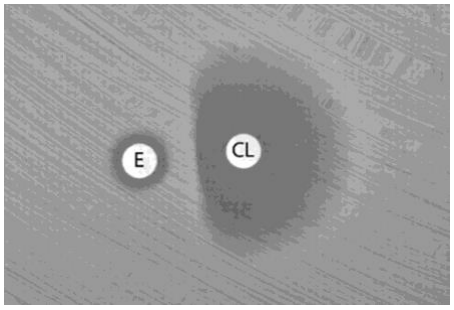
The prevalence of this phenomenon varies by location.

Most automated systems have an inducible clindamycin phenotype detection well and therefore inducible resistance detection will only be an issue for laboratories that use disc diffusion as their primary susceptibility testing methodology.

Inducible clindamycin resistance can be detected by antagonism of clindamycin activity by a macrolide agent using the D-test.

D-test:

Place the erythromycin and clindamycin disks 12-20 mm apart (edge to edge) and look for antagonism (the D phenomenon) to detect inducible clindamycin resistance.



Phenotype: requiring D-test.

Erythromycin	Clindamycin	D-test
R	S	Required

EUCAST: If inducible resistance is detected, then report as resistant and consider adding this comment to the report: "Clindamycin may still be used for short-term therapy of less serious skin and soft tissue infections as constitutive resistance is unlikely to develop during such therapy".

Resistance classification

Erythromycin	Clindamycin	D-test	
R	R	Not required	Constitutive Resistance
R	S	Positive	Inducible Resistance
R	S	Negative	Clindamycin Susceptibility

For the purposes of SNAP:

1) Enrolment into the clindamycin adjunctive therapy domain will be considered irrespective of susceptibility testing with analysis performed based on resistance classification.
2) Step-down to PO clindamycin therapy in the early oral switch domain is an inappropriate option in the presence of inducible resistance irrespective of the source of bacteraemia.

7.4. Daptomycin susceptibility Testing

Daptomycin MICs must be determined in the presence of Ca^{2+} (50 mg/L in the medium for broth dilution methods; agar dilution methods have not been validated). Follow the manufacturers' instructions for commercial systems.

For the purposes of SNAP: Consider performing daptomycin testing on multiple isolates especially in the setting of previous vancomycin therapy due to the co-selection of hetero-resistant VISA and DAP-R isolates.

7.5. hVISA Testing

No validated easy to perform methodology exists for the detection of heteroresistant VISA isolates. This phenomenon has only been described, to date, in high vancomycin MIC (~2μg/L) multi-resistant MRSA infections in the setting of vancomycin treatment failure. Moreover, these lineages usually represent hospital-adapted clones and have been restricted to MLST ST293 and ST5 types.

For the purposes of SNAP: No additional or specialised testing is required.

7.6. Repeated susceptibility testing[4,5]

There are no established recommendations on repeated susceptibility testing in the setting of persistent bacteraemia. IDSA guidelines suggest reassessing treatment if bacteraemia persists after 7 days, earlier in case of clinical deterioration. The risk of secondary resistance varies according to the backbone used (highest for daptomycin, lowest for flucloxacillin and cefazolin) and the bacterial burden (highest for endocarditis and implant-associated infections).

For the purposes of SNAP: Repeated susceptibility testing is recommended in case of persistent bacteraemia after ≥ 5-7 days of treatment with vancomycin or daptomycin. Due to the high prevalence of co-resistance, both vancomycin and daptomycin MIC testing should be performed. No specific

testing for oxacillin or cefazolin is required, however, superinfection with MRSA should be excluded using the approach described above. Note that a change beta-lactam sensitivity can also reflect the acquisition of BORSA mutations as described above. At least one blood culture isolate from persistent bacteraemia should be stored and sent to the reference lab.

7.7. Susceptibility Testing for Oral Agents

It is recommended that any oral agent chosen for therapy in the early oral switch domain test susceptible by a validated method. Several oral antibiotics would also require additional testing to confirm susceptibility. These include amoxicillin (to exclude penicillinase activity); and clindamycin (to exclude inducible resistance). Laboratories should follow their in-house testing and reporting protocols. Several oral agents have a low resistance barrier and should therefore not be used as monotherapy (see the early oral switch protocol for further details).

7.8. General Comments

Mixed infections:
For participants that are enrolled into the MRSA domain based on the molecular testing and subsequent growth reveals mixed infections with MSSA and a coagulase-negative *Staphylococcus* species. These participants should have their treatment modified as per the treating team. These participants will be included in the intention to treat analysis for the MRSA silo but be excluded from per-protocol analyses.

Changing antibiograms during therapy
For patients that are enrolled into a certain treatment domain e.g. PSSA and are randomised to receive penicillin. Subsequent blood cultures remain positive but are now resistant to treatment prescribed. These participants should have their treatment modified as per the treating team. These participants will be included in the intention to treat analysis for the relevant silo and the reason for change in treatment recorded.

8. REFERENCES

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2. Kaase M, Lenga S, Friedrich S, et al. Comparison of phenotypic methods for penicillinase detection in *Staphylococcus aureus*. Clin Microbiol Infect 2008;14(6):614-6.
3. Hryniewicz MM, Garbacz K. Borderline oxacillin-resistant *Staphylococcus aureus* (BORSA) - a more common problem than expected? J Med Microbiol 2017;66(10):1367-1373.
4. Liu C, Bayer A, Cosgrove SE, et al. Clinical practice guidelines by the Infectious Diseases Society of America for the treatment of methicillin-resistant *Staphylococcus aureus* infections in adults and children. Clin Infect Dis 2011;52(3):e18-55.
5. Humphries RM, Pollett S, Sakoulas G. A current perspective on daptomycin for the clinical microbiologist. Clin Microbiol Rev 2013;26(4):759-80.
6. Giulieri SG, Guérillot R, Kwong J, et al. Comprehensive Genomic Investigation of Adaptive Mutations Driving the Low-Level Oxacillin Resistance Phenotype in Staphylococcus aureus mBio. 2020 Dec 8;11(6):e02882-20.
7. Sommer A, Fuchs S , Layer et al. Mutations in the *gdpP* gene are a clinically relevant mechanism for β-lactam resistance in meticillin-resistant *Staphylococcus aureus* lacking *mec* determinants. Microbial Genomics 2021 Sep;7(9):000623.