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Development, optimization, standardization and validation of a simple in-house agar gradient method to determine vancomycin MIC's for Staphylococcus aureus

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Background: MIC determination has became easier, with introduction of the E-test. The point of contention has been the cost of the commercial strips which has restricted its use in resource limited countries. We attempted to develop, standardize and validate a simple in – house agar gradient method, to determine MIC of vancomycin for clinical isolates of *S. aureus*.

Methods & Materials: In – house strips were made from Whatmann filter paper no 1. The strips were impregnated with varying concentration of vancomycin solution so as to create an increasing antibiotic gradient along the strip. During the standardization step, MIC's of 90 clinical strains of *S. aureus* and ATCC 29213 were tested by the broth microdilution and commercial strip followed by the in – house strip. The results were kept blinded during the development stage. Variables for the preliminary considerations for optimization and standardization of the In – House Agar Gradient strip were considered to correct the outliers. This was followed by validation stage where MIC's of 90 different clinical strains of *S. aureus* and ATCC 29213 were determined by the in – house and results were kept blinded. This was followed by determination of MIC's by broth microdilution and commercial strips. An MIC reading of ± 1log2 dilution compared with broth microdilution was considered as an outlier

Results: During the optimization and standardization stage there were 7/90 outliers in the clinical strains and no outliers seen with the ATCC 29213 control strain. Corrective action was performed by increasing precaution during the antibiotic solution impregnation stage. During the validation stage, only 4/90 outliers were observed in the clinical strains. The commercial strips had 29/90 among clinical and 15/30 outliers in the control strain during the prevalidation phase. The supplier was informed to maintain cold chain and during the validation phase the outliers for commercial strip were 18/90 and 4/30 in the clinical and control strain respectively.

Conclusion: Vancomycin sensitivity is reported as MIC. Expensive commercial strips can be replaced by in – house strips using this simple technique after validating it with a gold standard method like broth microdilution.

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Multidrug-resistant acinetobacter baumannii – plasmid-borne carbapenem and aminoglycoside co-resistance causing outbreak in Southwest Virginia

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Background: Multidrug-resistant (MDR) Gram-negative bacterial pathogens, often resistant to more than one class of antibiotics including carbapenems and aminoglycosides, pose serious threats in healthcare settings worldwide. A 2015 White House task force issued guidelines to combat antibiotic-resistant strains in the United States, which the Center for Diseases Center has termed "nightmare bacteria". MDR pathogens cause at least two million illnesses and 23,000 deaths annually in the U.S. MDR *A. baumannii (MDR-Ab)* cause 10% of hospital-acquired infections (HAI) with 70% patient mortality. A mini-outbreak of carbapenem-resistant (CR) MDR-*Ab* (CR-MDR-*Ab*) occurred during a 2009-2010 H1N1 epidemic at Carilion Medical Center (CMC) in Virginia, U.S; three of the nine patients died. To develop effective strategies for prevention, control and treatment of MDR infections, we are performing whole genome analysis of the clinical isolates of CR-MDR-*Ab*.

Methods & Materials: To date, we have analyzed 68 *Ab* clinical isolates, including five CR-MDR-*Ab* outbreak strains. We have sequenced and analyzed whole genomes of the following MDR-*Ab* isolates: an isolate from outbreak patients (CMC-MDR-*Ab*4); a carbapenem-sensitive isolate (CMC-MDR-*Ab*59); and a CR-MDR-*Ab* (CMC-MDR-*Ab*66) isolate from sporadic cases. The sequencing was performed on the PacBioRSII platform.

Results: The CR CMC-MDR-*Ab* strains were found to carry two plasmids, pCMCVT*Ab*1 and pCMCVTAb2. The latter is conjugative type and carried two transposons: *Tn2008-like*, containing a beta-lactamase gene (bla_{OXA23}) and conferring CR, and *a TnaphA6* element causing aminoglycoside resistance and further reducing treatment choices. Their chromosomes carried five *bla* genes and an *aphA1* gene. A PCR analysis based upon the resistant determinants showed that all outbreak isolates (100%) carried pCMCVT*Ab*2 containing the two transposons. Fourteen of the remaining 63 isolates (22.22%) carried pCMCVT*Ab*2 and of these, six (42.85%) carried *Tn2008-like*, and two (14.28%) carried *TnaphA6*. These differences are statistically significant using Fisher's exact, two-tailed $p = 0.0011$, $p = 0.0445$ and $p = 0.0018$.

Conclusion: The results suggested that pCMCVTAb2 was responsible for the CR-MDR outbreak. A PCR analysis based on the antibiotic-resistant genes of pCMCVT*Ab*2 could be used for rapid identification of CR-MDR-*Ab* strains, thereby helping to prevent the

spread and providing guidance for identification and treatment of hospitalized patients with CR-MDR-*Ab* infections.

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Biofilm formation by Staphylococcus species on exposure of sub-lethal concentration of vancomycin

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Background: Staphylococcus is responsible for community acquired and nosocomial infection in different sites of human body. Drug resistant Staphylococcus such as Methicillin resistant Staphylococcus (MRSA) and vancomycin resistant *Staphylococcus aureus* (VSRA) has become common concern. The drug resistance increases 10 to 1000 folds when there is production of biofilm. This study has assessed level of biofilm production without and on exposure to sublethal concentration of vancomycin against the clinical isolates of staphylococcus.

Methods & Materials: A total 103 pure growth of staphylococci isolated at Department of Microbiology, Tribhuvan University Teaching Hospital (TUTH), Kathmandu, was included in the study. Of them 25 were MSSA, 25 MRSA, 23 MS-CONS and 30 MR-CONS. All isolates were subjected for determination of MIC of vancomycin by following the standard methods (**Creasten et al** and modified by **Stepanovic et al,2007**)

Results: Among 103 isolates of *Staphylococcus* species, many (97.1%) were found to have MIC of vancomycin within susceptible range, with few (2.9%) were found to have MIC of intermediate range. For *Staphylococcus aureus* 24.0% had MIC level of 2mg/l and for CONS 45.2% had the MIC level of ≥2mg/l showing high number of isolates towards upper limit of susceptible range of Methicillin resistant isolates.

Among the isolates, 63.1% of *Staphylococcus* species were producing different degree of biofilm with CONS sharing the larger percentage. Sub-lethal concentration of vancomycin has significantly ($p<0.05$) induced biofilm formation in both MRSA and MSSA, however, induced effect seems to be higher in MRSA isolates. On the other hand, in cases of CONS, sub-lethal concentration of vancomycin could not show significant induced effect ($p>0.05$)

Conclusion: This study has concluded that the MIC value of clinical isolates for Staphylococcus is increasing and the sublethal dose of Vancomycin induces biofilm production and thereby producing VRSA. Therefore the MIC determination prior to therapy and proper dosing should be done.

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Influence of variation in the sequence(s) of factors essential for methicillin resistance (fem genes) on the expression of resistance to Lysostaphin and secretion of DNAse

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Background: In *S. aureus*, cell wall consists of pentaglycine bridge which is catalyzed through *fem* (*f*actors *e*ssential for *m*ethicillin resistance) genes namely *fem*X, *femA and femB. Fem*X adds the 1st glycine, *fem*A adds the 2nd and 3rd glycine and *fem*B adds the 4th and 5th glycine of the pentaglycine bridge. The target of the lysostaphin is the pentaglycine cross-bridge between 3rd& 4th glycine. Mutations in any one of these *fem* genes, reportedly confer lysostaphin resistance but decreases resistance to beta-lactam compounds such as methicillin and oxacillin.

Methods & Materials: We screened all *Staphylococcus aureus* isolates (n=100) for expression of DNAase and screened 45 isolates which showed variations in *fem* gene sequences for the sensitivity of Lysostaphin. We determined the lysostaphin (20ug) susceptibility through broth dilution method (Hardy's Test) and DNAse by both methyl green agar method and also by PCR.

Results: Those *S.aureus* isolates which failed to amplify any of the *fem* genes, considered as total *fem* mutants displayed 80% resistance to lysostaphin when compared with wild isolates. Mutations in *fem*A alone resulted in 70%, 52% (*fem*B) and 52.7% (*fem*X) resistance to lysostaphin. *Fem* mutants which showed variable expression of DNAse were (*fem*A 2%; *fem*B 5%, *fem*X 2%) on DNAse agar, although all of these isolates were positive for DNAse PCR. When compared between the species specific markers ie; *nuc* gene and *fem* (A,B and X genes), *nuc* gene proved to be reliable for species identification

Conclusion: Lysostaphin cleaves the inter-glycine peptide bond between the 3rd and 4th glycine and it is expected that any variation in the *fem* A or *fem*B gene or *fem* X may have an impact on the synthesis of the pentaglycine bridge in the cell wall of *S.aureus*. Our results demonstrate that mutations in *fem* genes not only affect sensitivity to lysostaphin but also other cell wall functions like secretion of DNAse. We will also present results of analysis of these *fem* mutants on expression of PBP2a and the sensitivity to various antibiotics.

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