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Development, optimization, standardization, and validation of a simple in-house agar gradient method to determine minimum inhibitory concentration of vancomycin for *Staphylococcus aureus*

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Abstract:

BACKGROUND: The Clinical and Laboratory Standards Institute recommends reporting minimum inhibitory concentration (MIC) values of vancomycin for *Staphylococcus aureus*. Commercial MIC strips are expensive, and the traditional broth microdilution method is cumbersome. With this background, we attempted to develop and standardize an in-house agar gradient method to determine MIC values of vancomycin for *S. aureus*.

OBJECTIVES: To develop and validate an in-house vancomycin MIC strip, based on simple agar gradient method for *S. aureus* as per bioassay development guidelines.

MATERIALS AND METHODS: Filter paper gradient strips were made in house and impregnated with varying concentrations of vancomycin to create an antibiotic gradient. During standardization, MICs of ninety clinical strains of *S. aureus* and ATCC 29213 were tested by the broth microdilution and commercial strip followed by the in-house strip. During the validation stage, MICs of ninety different clinical strains of *S. aureus* and ATCC 29213 were determined by the in-house strip followed by MIC detection by broth microdilution and commercial strips. A reading of more than ± 1$\log_2$ dilution compared with broth microdilution was considered as an outlier.

RESULTS: During the initial stage, there were 7/90 outliers in the clinical strains, and no outliers were seen with the ATCC 29213 control strain. Corrective action included increasing precaution during the antibiotic impregnation on the strip. During 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. Despite maintaining cold chain during the validation phase, the outliers for commercial strip were 18/90 and 4/30 for clinical and control strains, respectively.

CONCLUSION: Reporting vancomycin MIC for *S. aureus* may be attempted using the in-house method after validating it with a gold standard broth microdilution method and quality control as per protocol.

Key words:

AMR, antimicrobial testing, Epsilometer test, in-house agar gradient method, minimum inhibitory concentration, vancomycin

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Introduction

Minimum inhibitory concentration (MIC) values of antimicrobials are considered to be better indicators of true inhibitory concentrations compared to zone diameters, especially for larger and poorly diffusing molecules such as vancomycin. The presence of heteroresistance in *Staphylococcus aureus* against vancomycin validates this preference, and the Clinical and Laboratory Standards Institute (CLSI) recommends that only MIC values should be reported for vancomycin for *S. aureus*. Determination of MIC has traditionally been performed by agar or broth dilution methods.[1] These standard reference methods are cumbersome and time-consuming. To make MIC determination easier, the PDM Epsilometer test was introduced by AB Biodisk, Solna, Sweden, which consists of a plastic strip with a predefined antimicrobial agent concentration gradient immobilized on one side and a continuous MIC scale covering 15 serial dilutions on the opposite side. The concept is that of instilling increasing volumes of a known amount of an antibiotic along a strip so that drops of impregnated antibiotic solution do not mix with each other, simulating many tiny antibiotic discs of increasing drug concentration placed closely along a strip. This, therefore, creates a uniform antimicrobial concentration gradient along the strip. After overnight incubation, the interaction of the antimicrobial agent gradient and the test bacterial strain produces elliptical inhibitory zones. The intersection of the growth ellipse margin with the plastic gradient strip indicates the MIC of the drug for the isolate.[2] This development has revolutionized antimicrobial sensitivity testing.

The only drawback has been the cost of the commercial strips which are now available from various manufacturers. This has been the chief factor limiting its use in resource-limited countries like India, where the average cost for one strip varies from US $1.5 for Indian manufacturers to US $4 for overseas manufacturers. This limitation becomes especially important for those antibiotics where breakpoints are available only as MIC values, such as vancomycin, colistin, and netilmicin.[3] Variable shelf life and breaks in the cold chain during transportation may also result in erroneous MIC values in the case of commercial strips.[4] Therefore, we attempted to develop, optimize, standardize, and validate a simple in-house agar gradient method, to determine the MIC of vancomycin for clinical isolates of *S. aureus*.

When an existing assay is being modified, prior knowledge, planning, and a study design are required. The purpose of development of the agar gradient strip was to determine the MIC of vancomycin against *S. aureus*. The in-house strip should fulfill the quantitative bioassay validation criteria, as the final result, i.e. the MIC value, and its interpretation will affect the clinical decision.[5] Different variables that were likely to affect the final interpretative reading and, therefore, the clinical decision had to be addressed.

During development of an infectious disease bioassay, three categories of variables must be assessed. The first category of variables includes the type of the sample, which are bacterial isolates in this case. The second category includes the type of system, which is the agar antibiotic gradient strip. The third category of variables includes the interpretation of the MIC result. Table 1 highlights the different variables that may affect the development, validation, and reproducibility of the in-house agar gradient strip and the preventive actions which were taken to minimize these variations.

Next, the criteria for assay validation had to be addressed. These include repeatability, analytical and diagnostic sensitivity, specificity, and reproducibility. The CLSI MIC interpretative criteria for vancomycin against *S. aureus* and *S. aureus* ATCC 29213 were used as the thresholds and cutoffs in this case.

Materials and Methods

Making the strips

Whatman filter paper no. 1 sheets were cut first into a standard A4 size sheet and fed into a laser printer and labeled "Va" to indicate the antibiotic for which the strip was being made. Templates of the strip, 75 mm long and 8 mm wide, were drawn with a pencil on these A4 sheets of Whatman filter paper. Lines were made along the strip at a distance of 9 mm. Seven holes of 5-mm diameter, using a stationery paper punch, were made over the lines so as to create eight sites for impregnating the antibiotic solution [Figure 1]. The purpose of creating seven holes in between the eight solution impregnation sites was to create separate gradients and prevent the different concentration solutions from mixing together at the time of impregnating the antibiotic solution. These paper strips were then cut and sterilized in a hot air oven.

Creating the antibiotic gradient on the strips

The exact volume of solution to be impregnated between the holes had to be determined first. Black monochrome liquid ink was impregnated between the holes, and it was observed that no mixing of ink blots occurred when 5 µL of the liquid was used as the volume to be delivered on the strip. Therefore, we decided to use 5 µL of antibiotic solution for strip impregnation between the holes. Considering the vancomycin breakpoint testing range for *S. aureus* according to the CLSI, 16 mg of pure vancomycin powder (HiMedia, HiMedia Laboratories Pvt Ltd, Mumbai, Maharashtra, India) was dissolved in 5 mL of sterile double-distilled water so as to have

Table 1: Variables for the preliminary considerations for optimization and standardization of the in-house agar gradient strip

Variations	Preventive actions taken to rule out variations
The sample: Bacterial strains including the control strain ATCC 29213 and test isolates	
Storage of reference strain	ATCC 29213 was maintained and revived as per the CLSI protocol
Purity and age of isolate	Pure isolates from fresh overnight growth from nonselective media were used
Identification	Test strains of *Staphylococcus aureus* were identified by standard identification tests[6]
Bacterial inoculum	Bacterial inoculum with turbidity matched to that of 0.5 McFarland commercially available turbidity standard was used for broth microdilution, commercial, and in-house agar gradient strips as per the CLSI and manufacturer's instructions
The assay system: The in-house MIC strip on Mueller Hinton agar	
Strip dimensions	The length, width, and holes were made at equal dimensions. Thinner, thicker strips or those with holes made at unequal distances were discarded
Antibiotic impregnation	
Solvent quality	Double-distilled sterile (autoclaved) water was used
Antibiotic powder	Vancomycin powder was acquired from the WHO GMP, CE, and ISO 13485-certified company
Quantity	Calibrated electronic scale and pipettes used
Pipetting errors while impregnating the strip	If the volume of the antibiotic solution in all the eight tips was not equal at the time of aspiration of the antibiotic solution, the tips were readjusted to fit properly to avoid air entry during aspiration. If the impregnated drops were of unequal size after diffusion on the strip, the strip was discarded
Storage	Up to ten strips were stored in universal container with rubber washer and a silica desiccator at 2°C–8°C for up to 14 days
Shelf life	Being in-house strips, with all the components readily available, shelf life beyond 14 days was not evaluated
Result interpretation: MIC values	
Observer variations	Specific objective parameters were defined for reading the breakpoint MICs for the in-house strips
	All MIC readings on the in-house strip were taken by one observer only, and parameters were followed to rule out intraobserver variation
Incubation conditions	35°C±2°C in ambient air for 24 h

CLSI=Clinical laboratory standards institute, GMP=Good manufacturing practices, CE=Conformité européene, ISO=International organization for standardization, MIC=Minimum inhibitory concentration

16 μg of antibiotic per 5 μL of solution. 300 μL of this 16 μg/5 μL stock solution was taken in the first well of a sterile U-bottomed microtiter plate and 150 μL of sterile double-distilled water in subsequent wells. 150 μL of the stock was taken from the first well, and doubling dilutions were made sequentially so that the first to last well had a concentration from 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 μg per 5 μL [Figure 2a and b]. The sterile filter paper strips were then carefully impregnated in the center between the holes with 5 μL of the antibiotic solutions of varying concentrations from the microtiter plate, using a multi-channel pipette with tips exactly 9 mm apart, thereby creating an antibiotic gradient on the strip [Figure 3].

Performing minimum inhibitory concentration using broth microdilution, commercial, and in-house minimum inhibitory concentration strips

Broth microdilution for vancomycin was performed for *S. aureus* ATCC 29213 and clinical isolates of *S. aureus* as per the CLSI guidelines using the same MIC range as that of the in-house strips, i.e. from 0.125 to 16 μg/mL.[1] Fresh bacterial suspensions of 0.5 McFarland turbidity were made in Mueller Hinton broth and used for all the three assays to minimize inoculum variation. The tubes and plates were incubated at 35°C for 24 h. Readings for commercial strips, broth microdilution, and in-house strips were taken by separate, blinded observers to avoid bias. MIC reading of the commercial strip was taken as per manufacturer's instruction and for broth microdilution as per the CLSI guidelines.[1] MIC readings of the in-house strips were taken similarly to the commercial agar gradient strips, i.e. the intersection of the elliptical inhibitory zones produced after overnight incubation with the gradient strip indicated the MIC of the drug for the isolate. The following parameters were considered for reading the MICs for the in-house strips:

1. If the intersection was below the line separating two concentrations, the higher concentration was considered the MIC [Figure 4a]
2. If the intersection on either side was not at the same line, the concentration above the higher line was considered the MIC [Figure 4b]
3. If there was growth in hole separating the two concentrations, the higher concentration was considered as the MIC [Figures 4a and b].

The in-house test and commercial test results were taken to be accurate if the result was within ±1 $\log_2$ dilution

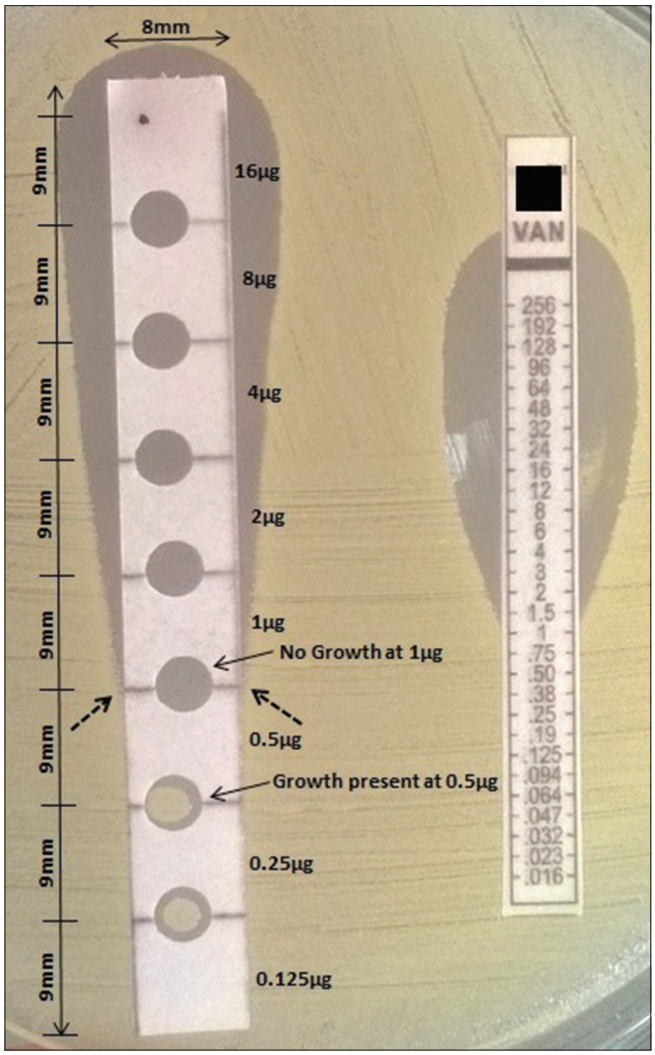

Figure 1: The dimensions of the in-house strip and its relative size compared to the commercially available strip placed together on the same plate to avoid inoculum variation

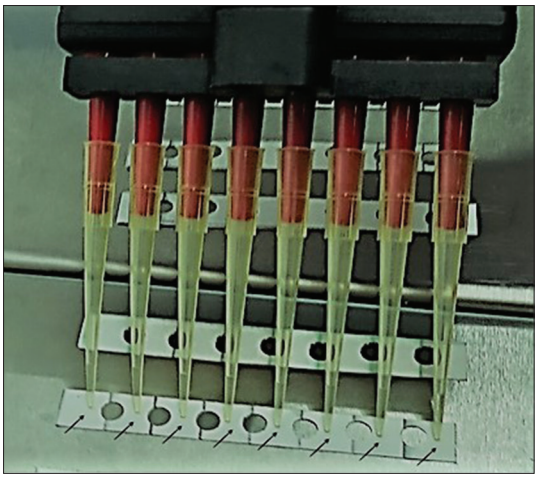

Figure 3: How the impregnation was done exactly at the center (arrows) of each point on the strip

of the reference method, i.e. broth microdilution method.[7]

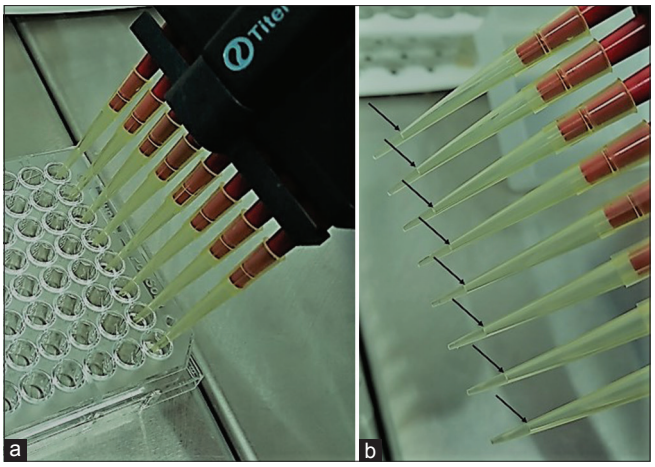

Figure 2: How the 8-channel micropipette was used to aspirate 5 µL of antibiotic solution to create a gradient along the strip as in (a) and visual confirmation was done to check equal volume being impregnated on every spot of the strip (arrows) as in (b)

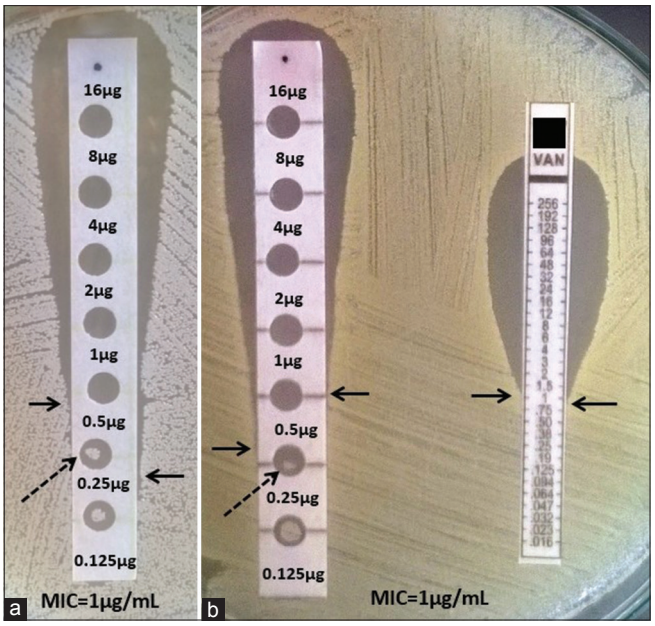

Figure 4: (a and b) How to take the reading of the strip. If there was growth in hole separating the two breakpoints, the higher breakpoint was considered as the breakpoint minimum inhibitory concentration as in a, there is growth adjacent to 0.5 µg/mL; therefore, the minimum inhibitory concentration is 1 µg/mL. Similar is the case with b

Initial test optimization and standardization

MICs of 90 clinical isolates of *S. aureus* were tested. In each test run, nine clinical isolates of *S. aureus* were tested along with three replicates of *S. aureus* ATCC 29213 reference strain, thereby completing the 12 rows of the microtiter plates as well as 12 in-house strips. One in-house and one commercial strip were placed on one 90-mm plate of Mueller Hinton agar, for better comparison and to rule out plate-to-plate variation. Therefore, the bioassay optimization and standardization process included ninety clinical strains and a total of thirty replicates of ATCC 29213 completed over ten runs.

For both broth microdilution and in-house strip, the MIC range included the sensitive and resistant breakpoint MICs of vancomycin for *S. aureus* as per the CLSI, i.e., from 0.125 to 16 μg/mL as eight doubling dilution readings. The results for in-house and commercial strips and for broth microdilution were recorded by different observers blinded to results of the other tests [Table 2].

The root cause analysis of outliers, i.e., MICs outside $\pm 1\log_2$ dilutions, was done, and faulty impregnation of the in-house strip was found to be the major cause. Corrective and preventive actions included airtight attachment of tips, equal aspiration of solution in all tips, and impregnation at the center of the area between the holes.

Post standardization validation

After optimization of the in-house strip, validation was performed on ninety different clinical isolates and ATCC 29213 control strain similar to the optimization and standardization protocol. MICs of clinical and control strains was determined using the in-house and commercial agar gradient strip, and the results were recorded by one observer. This was followed by determination of MIC of these strains using the reference broth microdilution method, and the results were recorded by another observer blinded to the result of the in-house strip. For quantitative bioassays, the analysate (bacterial MIC in this case) has to be tested at low, intermediate, and higher values. Since vancomycin resistant strains are not available for *S. aureus*, twenty vancomycin-resistant enterococcal isolates were used to evaluate higher vancomycin MICs.

Results

The MICs determined by different methods during the two stages of the study are displayed in Table 2 (Raw Data). During the standardization and optimization stage, MICs for ninety clinical isolates were determined, and MIC of *S. aureus* ATCC 29213, reference strain, was evaluated in triplicate for all the three methods, every tenth test run. Zero error indicated reading same as that of broth microdilution method. Minor but acceptable error was considered when nonconcordance was observed among MIC reading with a difference of $\pm 1\log_2$ dilutions between the in-house MIC strip and the commercial strip against broth microdilution. A difference of equal to or more than $\pm 1\log_2$ was considered as the major error. The nonconcordance is summarized in Table 3. During the standardization and optimization stage, the concordance was 83/90 (92.2%) for in-house strips and 60/90 (66.67%) for the commercial strips, against the gold standard broth microdilution method of the clinical strains. After root cause analysis and performing the corrective action and preventive actions of careful strip impregnation and better standardization of bacterial inoculum, the concordance rate improved to 86/90 (95.5%) and 70/90 (77.78%) for in-house and commercial strips, respectively. However, the concordance rate was 30/30 (100%) for the ATCC 29213 control strains during both the stages.

The MICs of all the twenty VRE isolates were more than 16 μg/ml by all the three methods. There were multiple instances where the commercial strips gave MICs of 2, 4, and 3 μg/mL, whereas the in-house and broth microdilution gave results in the susceptible range [Figures 5a and b]. There were instances where the drop could not be impregnated exactly at the center, and there was mismatch between the elliptical zone meeting the strip on either side. In such case, the higher MIC was taken as the final reading as done for commercial strips [Figure 6a and b].

Discussion

With the advent of pharmacokinetics (PK)- and pharmacodynamics (PD)-based customization of antimicrobial therapy, under stewardship programs, provision of antimicrobial test results as MICs makes more sense than reporting qualitative interpretations.[8] This is because time-dependent antimicrobials, given by slow infusion, have been able to produce bacterial killing even in intermediate resistant or resistant strains with suitable MICs.[9,10] Nonavailability of breakpoint zone sites for vancomycin makes it mandatory to report MICs of vancomycin. The PK/PD parameter used for the optimum use of vancomycin is the total amount of drug which is the 24 h area under the curve value to the MIC ratio (AUC_{24}/MIC), and this value must be above 400. This further validates the importance of reporting MICs for vancomycin.

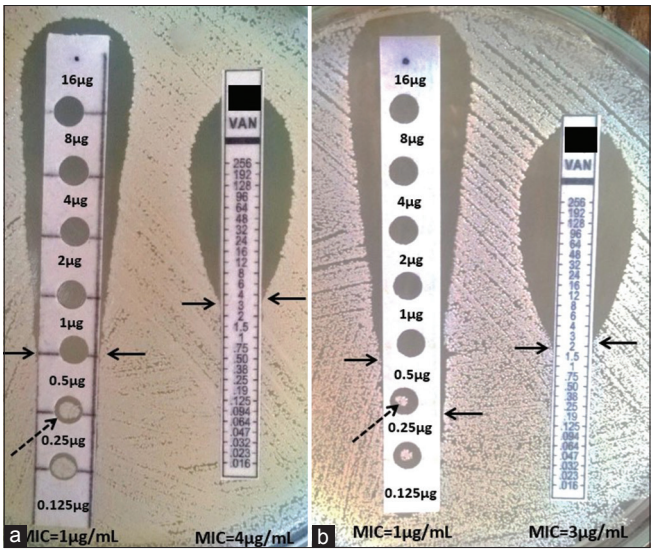

Figure 5: (a and b) Gross discrepancy among minimum inhibitory concentration values from the commercial strip (higher) against the in-house strip

Table 2: Highlighting data of in-house agar gradient strip optimization and standardization with broth microdilution used a standard reference method followed by poststandardization validation results

Optimization and standardization result				Poststandardization validation result			
Strain	Broth microdilution MIC	Epsilometer test (HiMedia)	In-house strip	Strain	In-house strip	Epsilometer test (HiMedia)	Broth microdilution MIC
ATCC 29213	0.5, 0.5, 0.5	1, 1, 0.5	0.5, 1, 0, 5	ATCC 29213	0.5, 0.5, 1	0.5, 1, 0.75	0.5, 0.5, 0.5
P459May2014	0.5	2*	0.5	P306Jun2014	1	1.5	1
P464May2014	0.5	2*	1	P312Jun2014	1	1.5	1
P467May2014	1	4*	4*	P322Jun2014	0.5	1	0.5
P468May2014	1	3*	1	P330Jun2014	1	1.5	1
P470May2014	1	2	0.5	P337Jun2014	0.5	1.5	1
P474May2014	1	4*	1	P345Jun2014	1	2*	0.5
P483May2014	0.5	3*	2*	P367Jun2014	1	1	1
ATCC 29213	0.5, 0.5, 0.5	2, 1, 2*	1, 1, 1	P371Jun2014	1	1.5	1
P485May2014	2	3	0.5*	P379Jun2014	2*	1.5*	0.5
P486May2014	1	3*	2	ATCC 29213	0.5, 0.5, 0.5	1, 1, 1.5*	0.5, 0.5, 0.5
P491May2014	2	4	1	P385Jun2014	1	1.5	1
P497May2014	2	3	1	P390Jun2014	1	1.5	1
P498May2014	0.5	4*	1	P395Jun2014	0.5	1	0.5
P507May2014	0.5	4*	1	P398Jun2014	1	1.5	1
P516May2014	1	2	1	P402Jun2014	1	1.5	1
P601May2014	1	2	1	P408Jun2014	1	1.5*	0.5
P610May2014	1	4*	1	P415Jun2014	0.5	1.5	1
P618May2014	0.5	4*	1	P419Jun2014	0.5	1	0.5
P619May2014	2	4	1	P423Jun2014	0.5	1	1
P622May2014	1	3*	1	ATCC 29213	1, 0.5, 1	1, 1, 0.5	0.5, 0.5, 0.5
P639May2014	2	4	1	P438Jun2014	1	1	1
P642May2014	2	3	1	P443Jun2014	1	1.5	1
P643May2014	2	2	1	P451Jun2014	0.5	1.5*	0.5
P644May2014	2	2	1	P459Jun2014	1	1.5	1
U1269May2014	2	2	1	P464Jun2014	1	1.5	1
ATCC 29213	0.5, 0.5, 0.5	3, 2, 3*	1, 1, 1	P473Jun2014	1	1.5*	0.5
P676May2014	0.25	1.5*	1*	P482Jun2014	0.5	1.5*	1
P703May2014	1	0.75	4*	P491Jun2014	0.5	2*	0.5
P704May2014	2	1	0.5*	P501Jun2014	0.5	1	1
P708May2014	1	1	1	ATCC 29213	0.5, 1, 0.5	1, 0.5, 0.5	0.5, 0.5, 1
P710May2014	0.5	1	1	P508Jun2014	1	1	1
P711May2014	1	1.5	1	P512Jun2014	0.5	1.5*	0.5
P717May2014	0.5	1.5*	1	P518Jun2014	1	1.5	1
P718May2014	0.5	1	1	P523Jun2014	1	1.5*	0.5
P720May2014	0.5	1.5*	1	P530Jun2014	0.5	2*	0.5
P723May2014	1	1	0.5	P536Jun2014	1	2	1
P724May2014	0.5	3*	1	P542Jun2014	1	2	1
P729May2014	1	3*	1	P547Jun2014	0.5	1	0.5
P730May2014	0.5	3*	1	P548Jun2014	0.5	1	0.5
P731May2014	2	2	1	ATCC 29213	0.5, 0.5, 0.5	0.5, 1, 1	0.5, 0.5, 0.5
P735May2014	1	3*	1	P555Jun2014	1	0.5	1
P740May2014	1	3*	1	P561Jun2014	1	1	1
P741May2014	0.5	3*	1	P569Jun2014	0.5	1	1
ATCC 29213	0.5, 0.5, 0.5	2, 1.5, 1.5*	0.5, 0.5, 0.5	P574Jun2014	0.5	1	0.5
P744May2014	1	3*	1	P578Jun2014	0.5	1.5*	0.5
P786May2014	1	2	1	P589Jun2014	1	1	1
P793May2014	1	1	1	P594Jun2014	1	1	1
P795May2014	1	1	1	P595Jun2014	0.5	1	1
P798May2014	0.5	0.75	1	P599Jun2014	0.5	0.5	0.5
ATCC 29213	0.5, 0.5, 0.5	2, 2, 1.5*	0.5, 0.5, 1	ATCC 29213	1, 1, 0.5	1, 1, 0.5	1, 0.5, 0.5
P787Jun2014	1	1	0.5	P609Jun2014	1	1	1

Contd...

Table 2: Contd..

Optimization and standardization result				Poststandardization validation result			
Strain	Broth microdilution MIC	Epsilometer test (HiMedia)	In-house strip	Strain	In-house strip	Epsilometer test (HiMedia)	Broth microdilution MIC
P802Jun2014	1	4*	1	P615Jun2014	1	1	1
P805Jun2014	1	1	1	P616Jun2014	1	1	0.5
P807Jun2014	2	2	1	P620Jun2014	1	1	1
P816Jun2014	2	3	1	P629Jun2014	0.5	1	0.5
ATCC 29213	0.5, 0.5, 0.5	1, 1.5, 1.5*	0.5, 0.5, 0.5	P637Jun2014	0.5	1	0.5
P1Jun2014	1	1.5	1	P641Jun2014	1	2	1
P2Jun2014	1	1.5	1	P649Jun2014	0.5	1	1
P3Jun2014	2	2	1	P654Jun2014	0.5	1	1
P4Jun2014	2	2	1	ATCC 29213	1, 0.5, 0.5	1, 1, 1.5*	0.5, 0.5, 0.5
P5Jun2014	2	3	1	P655Jun2014	0.5	0.5	0.5
P24Jun2014	1	2	0.5	P656Jun2014	1	1	0.5
P25Jun2014	0.5	1	1	P663Jun2014	0.5	1	0.5
P27Jun2014	1	1	1	P669Jun2014	1	2	1
P29Jun2014	1	0.75	1	P673Jun2014	1	0.5	1
P30Jun2014	1	1.5	1	P681Jun2014	0.5	1	0.5
ATCC 29213	0.5, 0.5, 0.5	1, 0.5, 1	0.5, 0.5, 0.5	P689Jun2014	1	1	1
P34Jun2014	2	2	0.5*	P707Jun2014	1	1	1
P36Jun2014	1	1.5	2	P709Jun2014	0.5	1	0.5
P63Jun2014	2	3	1	ATCC 29213	0.5, 0.5, 1	1, 1, 2*	0.5, 0.5, 0.5
P64Jun2014	1	2	1	P714Jun2014	0.5	1	1
P65Jun2014	2	1.5	1	P720Jun2014	1	2	0.5
ATCC 29213	0.5, 0.5, 0.5	1, 1, 1.5*	0.5, 0.5, 0.5	P724Jun2014	0.5	2*	0.5
P71Jun2014	1	1.5	0.5	P729Jun2014	0.5	1	0.5
P72Jun2014	1	3*	0.5	P736Jun2014	0.5	1	0.5
P74Jun2014	1	2	1	P741Jun2014	0.5	1	0.5
P75Jun2014	1	2	1	P747Jun2014	0.5	2	1
P86Jun2014	1	0.75	0.5	P771Jun2014	1	2	1
P89Jun2014	2	1.5	1	P773Jun2014	1	2	1
P92Jun2014	1	1.5	0.5	ATCC 29213	1, 0.5, 1	1, 0.5, 0.5	0.5, 0.5, 0.5
P94Jun2014	1	1.5	0.5	P779Jun2014	0.5	1	1
ATCC 29213	0.5, 0.5, 0.5	0.75, 1, 1.5*	0.5, 1, 1	P783Jun2014	1	2	1
P99Jun2014	1	1	1	P789Jun2014	2*	2*	0.5
P102Jun2014	1	1	1	P794Jun2014	1	2*	0.5
P103Jun2014	2	1.5	1	P801Jun2014	1	2*	0.5
P118Jun2014	1	1.5	1	P807Jun2014	1	1	1
P126Jun2014	2	0.5	1	P809Jun2014	1	1	1
ATCC 29213	0.5, 0.5, 0.5	1, 1, 1.5*	1, 1, 1	P813Jun2014	0.5	1	0.5
U175Jun2014	1	1	1	P817Jun2014	0.5	1	0.5
P131Jun2014	0.5	1	0.5	ATCC 29213	0.5, 0.5, 0.5	1, 0.75, 0.5*	0.5, 0.5, 1
P148Jun2014	1	1.5	1	P823Jun2014	2*	1	0.5
P149Jun2014	0.5	0.5	1	P825Jun2014	1	1.5	1
P154Jun2014	0.5	1.5*	0.5	P829Jun2014	0.5	1.5*	0.5
P159Jun2014	0.5	1.5*	1	P833Jun2014	1	1	1
P178Jun2014	1	2	0.5	P840Jun2014	1	2*	0.5
P184Jun2014	1	3*	1	P844Jun2014	1	2	1
P192Jun2014	0.5	2*	0.5	P848Jun2014	2*	1	0.5
P197Jun2014	0.5	1.5*	1	P851Jun2014	1	1	1
P202Jun2014	1	2	0.5	P852Jun2014	1	2*	0.5

All values are in µg/ml. MIC=Minimum inhibitory concentration

Commercial agar gradient plastic or paper strips are available, but cost limits their use. The per unit cost calculated for the in-house strip was Rs. 8.5, including cost of consumables, equipment, calibration, maintenance, and workforce. The cost of commercial strips ranged from Rs. 70 to 80 for Indian and from Rs. 180 to 320 for oversees manufacturers. The precision of values produced by these commercial strips is

Table 3: Summary of concordant (zero error and minor error) and nonconcordant (major error) readings of the in-house and commercial minimum inhibitory concentration strips against broth microdilution reference method

	In-house strip			Commercial strip		
	Acceptability (%)		Outside±1log$_2$ dilution (major error)	Acceptability (%)		Outside±1log$_2$ dilution (major error)
	Same reading as broth microdilution (zero error)	Within±1log$_2$ dilution (minor acceptable error)		Same reading as broth microdilution (zero error)	Within±1log$_2$ dilution (minor acceptable error)	
Standardization and optimization stage (%)						
ATCC 29213	17/30	13/30	0/30	2/30	12/30	16/30
	30/30 (100)			14/30 (46.67)		
Clinical isolates	35/90	48/90	7/90	19/90	41/90	30/90
	83/90 (92.2%)			60/90 (66.67%)		
Validation stage (%)						
ATCC 29213	20/30	10/30	0/30	15/30	12/30	3/30
	30/30 (100)			27/30 (90)		
Clinical isolates	62/90	24/90	4/90	20/90	50/90	20/90
	86/90 (95.5%)			70/90 (77.78)		

MIC=Minimum inhibitory concentration

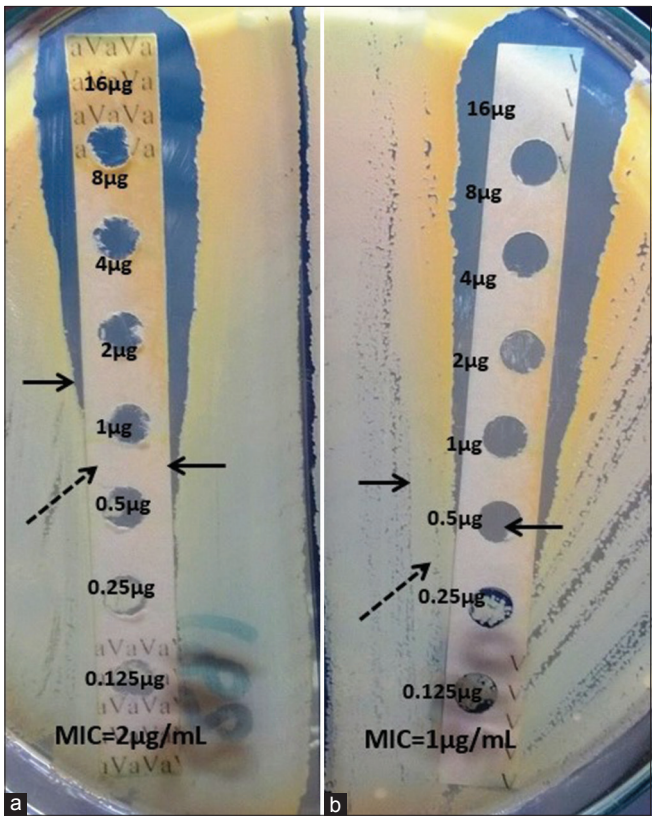

Figure 6: (a and b) When the impregnation technique was faulty, there were gross errors in the zones meeting on both sides

questionable as MIC values provided are 0.5–1.5 log$_2$ dilutions higher than broth microdilution as evident in the present work. The primary reason for this was the inability to maintain cold chain during transportation in tropical countries like India.[11] Commercial tests for broth microdilution with precoated antibiotic powders in microtiter plates are also available in the market, but cost is the major factor that restricts their routine use.[12] The in-house strips made in this study have their pitfalls as well. This method is an oversimplification of the commercial technology which can be applied in a diagnostic laboratory. However, it requires to be tested on a wider spectrum of bacterial strains and antimicrobials for further validation. Besides the lower cost, it cuts out the process of maintaining a cold chain as it can be made from fresh antibiotic stock solution.[11] This study needs to be further extended to include a greater number of clinical isolates and evaluated for shelf life and feasibility with other antimicrobials such as colistin for which only MIC values are allowed.[13]

Conclusions

To the best of our knowledge, this is the first original work where an in-house agar gradient strip has been developed, standardized, and validated for vancomycin. The feasibility of using the above-mentioned principle of in-house agar gradient strip was initially optimized and found to be suitable, and the validation was also found satisfactory. The most critical step is the impregnation of the strip with the exact amount of the antibiotic and to be put at the exact center of the designated area. If the drop gets impregnated on the side, it gave erroneous results and irregular ellipses. Therefore, if one is attempting to make such a strip, accurate micropipetting and impregnation is mandatory. However, we reiterate that if this method is used, it must be compared with broth microdilution method and adopted only after completing in-house validation protocol. The longevity and shelf life of such strip was not assessed as this could be made in house. If this technique is adopted, one must follow the quality control frequency cycles for MIC reporting as per the CLSI M-07.[1] This technique if correctly adopted and scientifically validated will not only cut down the cost of

procuring commercial strips, but will also provide true MICs rather than higher MICs as read by commercial strips. To bring the in-house strip into routine use, it needs to be further tested on multiple clinical isolates and this would require multiple trials. However, if used in its present state, the results must be compared and correlated with the gold standard broth microdilution method.

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Conflicts of interest
There are no conflicts of interest.

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