



## RAPID PHENOTYPIC DETECTION AND DIFFERENTIATION OF PATHOGENIC AEROBIC BACTERIA USING ALTERNATIVE METHODS TO STAINING.

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**ABSTRACT** **Background & aim:** The present study investigates the pathogenic aerobic bacteria isolated from different sources of clinical samples in a tertiary care hospital with the aim in proving that there are also couple of other methods for confirming the preliminary classification of bacteria without using Gram staining.

**Materials & methods:** The study has been carried out during December 2015 to March 2016. All the isolates were subjected to Gram staining, Vancomycin susceptibility test and KOH string test.

**Results:** Our prospective study showed that out of 268 isolates, 185 (69.02%) were gram negative bacilli and 83(30.97%) were gram positive cocci. Gram negative rods including *Escherichia coli*, *Klebsiella sp.*, *Pseudomonas sp.*, *Proteus sp.*, *Enterobacter spp.* and gram positive cocci included *Staphylococcus sp.*, *Streptococcus sp.* and *Enterococcus sp.* All gram negative bacilli shows 100% vancomycin resistance and 100% KOH test positive. On the other hand, all gram positive cocci show 89.15% vancomycin sensitive, 10.84% resistance and KOH string test is 100% negative.

**Conclusion:** The alternative test of Gram staining is Vancomycin susceptibility test and KOH test that easily identifies the gram negative and gram positive bacteria very well.

**KEYWORDS :** Gram negative bacilli, Gram positive cocci, Vancomycin, KOH string test.

### Introduction

The backbone of bacteriology starts with Gram staining. In order to initially identify & classify the unknown bacterium, we largely rely on these staining results which are essential step in both diagnostic microbiology and clinical medicine (8). But unfortunately due to certain circumstances & conditions the magical staining procedure fails us. Mostly the error in staining is human error as we tend to decolorize it; there are other factors also like preparation of staining reagents, composition of the medium, age of the culture etc., which affects the results (4).

Apart from decolourisation Gram variability is also a challenge to bring the conclusion. Several modifications of the Gram staining have been developed to overcome these practical difficulties. Gram staining fails due to following reasons like increasing acidity, increasing alkalinity, lipid covering etc. The Gram stain modifications are as follows: Kopeloff & Beermann's modification, Jensen's modification, Weigert's modification, Preston & Morrell's modification but still the decolourization becomes difficulty (3). Even though Gram staining has its own practical difficulties, it still stays as the golden key to open the doors of bacteriology. Fortunately recent studies reveals us of two more rapid methods for preliminary differentiation of bacteria into Gram positive & Gram negative using 3% of KOH (Potassium Hydroxide) and Vancomycin susceptibility test.

KOH & Gram staining are based on the difference in the chemistry of the bacterial cell wall. The cell wall of gram negative bacteria is easily disrupted when exposed to dilute alkali solution. Another method is by using Vancomycin disc sensitivity. This is a comparative study of the standard Gram staining with Vancomycin susceptibility test & potassium hydroxide string test. Present study highlights the rapid method to determine the pathogenic aerobic bacteria in different sources of clinical samples from a tertiary care hospital.

### Materials & Methods

#### Sample Size:

A total of 268 clinical isolates were collected from a tertiary care hospital during December 2015 to March 2016.

#### Isolation & Identification:

Clinical samples were inoculated in 5% Sheep blood agar, MacConkey agar, Nutrient agar and the plates were incubated at 37°C for 18–24 hrs. Plates were observed for growth. Then all the isolates were subjected to Gram staining, Vancomycin susceptibility test and KOH test.

#### Gram staining:

Bacterial smears were prepared using smooth saline suspension by heat fixing technique. In the 1<sup>st</sup> step, the bacterial smears were flooded with crystal violet for 1 minute and then washed gently in running tap water. In the 2<sup>nd</sup> step, smears were flooded with Grams iodine for 1 minute and then washed with tap water. In the 3<sup>rd</sup> step, slides were exposed to acetone for decolourisation & washed immediately with running tap water. Finally, dilute Carbol fuchsin was added as the counter stain & washed after 1 minute. After drying, stained slides were examined under oil immersion 100X objective to note Gram reaction, morphology and arrangement (2,10).

#### Vancomycin Susceptibility test:

The isolates were tested by Kirby Bauer disk diffusion method on Mueller Hinton Agar plates. 1-2 colonies from the culture plates were inoculated into 2ml nutrient broth and incubated at 37°C for 2 hours. Turbidity was compared to that of 0.5 McFarland's standard (1.5 x 10<sup>7</sup> CFU/ml). A cotton swab was immersed in the inoculum, the swab was then pressed to the slides to the tubes so on to remove excess inoculum. The swab was then used or preparing lawn culture in Mueller Hinton agar media. The Vancomycin antibiotic discs (5mg) were placed within 15 minutes on the lawn culture and were incubated at 37°C overnight. Interpretation was measured as any zone of inhibition (> 6mm) was considered vancomycin sensitive (2,5).

#### KOH – String Test:

Two drops of 3% KOH solution was placed in a microscopic slide. Loop full of bacterial cultures obtained from a 48 hours culture on blood agar was stirred in a circular motion in KOH solution. The loop was occasionally raised to 1-2 cm from the surface of the slide. The KOH solution characteristically became very viscous and mucoid with the Gram negative bacteria. A string of the mixture would follow the loop when it was raised. The KOH test was only considered positive if stringing occurred within the first 30 seconds of mixing the bacteria in the KOH solution. Gram positive bacteria suspended in the KOH solution generally displayed no reaction (2,5).

#### Results & Discussion

The first and the most important step in bacterial identification is the gram staining to differentiate bacteria into Gram positive & Gram negative bacteria. The present study brings out the alternative method to identify the Gram positive & Gram negative bacteria by KOH string test & vancomycin susceptibility test alternatives there will be reduced false positive and false negative results (6). With these A string is formed when an intracellular viscous or emulsified material is obtained as Gram negative bacterial cell wall is dissolved in 3% KOH solution and the DNA gets liberated forming a viscid material (7), where as gram positive cell wall does not dissolve. This KOH test is the rapid and

inexpensive experiment compared to Gram stain (9).

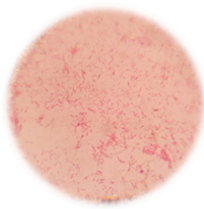


Fig: 1 Gram negative bacilli

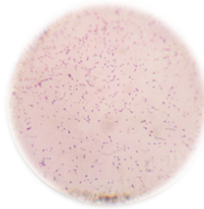


Fig: 2 Gram positive cocci



Fig: 3 Gram positive cocci showing zone of inhibition and Gram negative bacteria resistant to Vancomycin disc (5 mg)

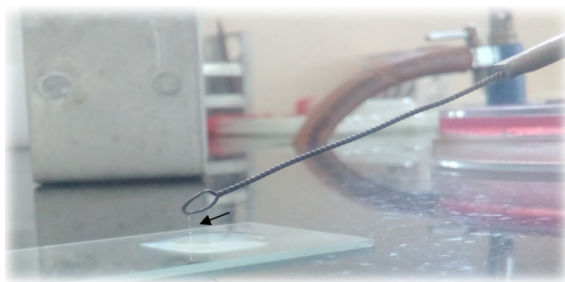


Fig: 4 Gram negative bacteria showing positive KOH string

In a study done by Arthi et al., (2003) 173 Gram negative bacteria (99.42%) were resistant to vancomycin while 1 (0.58%) *Acinetobacter calcoaceticus* was sensitive to vancomycin and 172 (98.85%) isolates was positive for KOH string test while 2 (1.15%) *Acinetobacter calcoaceticus* were negative for stringing this may be due to false positive result.

Another study 100% of Gram negative bacteria were positive for KOH and resistant to Vancomycin, 98.23% were Vancomycin sensitive and 100% negative for KOH test (11). Our study showed all the 185(100%) Gram Negative bacilli were Vancomycin resistant and also KOH positive. On the other hand out of 83 isolates 74 (89.15%) Gram Positive cocci were Vancomycin sensitive and 9 (10.84%) were resistant.

**Distribution of Gram Positive & Gram Negative isolates:**

Gram negative bacilli	No. of isolates	Gram positive cocci	No. of isolates
<i>Escherichia coli</i>	102	<i>Staphylococcus sp</i>	71
<i>Klebsiella sp</i>	68	<i>Streptococcus sp</i>	8
<i>Pseudomonas sp</i>	9	<i>Enterococcus sp</i>	4
<i>Proteus sp</i>	5		
<i>Enterobacter sp</i>	1		
Total	185	Total	83

**Results of Vancomycin Susceptibility Test:**

Different bacterial isolates	Vancomycin Susceptibility test		KOH test	
	SENSITIVE	RESISTANT	POSITIVE	NEGATIVE
Gram negative bacilli	0	185 (100%)	185 (100%)	0
Gram positive cocci	74 (89.15%)	9 (10.84%)	0	83 (100%)

Thus we can understand a highly significant association between these three tests: Gram Staining, Vancomycin sensitivity test & KOH test

**Conclusion**

KOH string test & Vancomycin susceptibility test are the alternative test to Gram staining in order to differentiate Gram Positive & Gram Negative bacteria which reduces the operator microscopic errors. However Gram staining stays as a golden standard in the field of bacteriology. Further studies have to be done for analysing the alternative methods for proving it as a standard method comparing to Gram staining method.

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