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Original Research Article

The Rapid and Easy Method to Detect the Pathogenic Aerobic Bacteria without Staining: A Case Study from Bankura and Its Surrounding Area, West Bengal, India

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Abstract: The present work investigates the pathogenic-aerobic bacteria in different sources of samples without staining in the community of Bankura and surroundings, during March 2015 to June 2015. In this study, all the isolates were subjected to gram staining, Vancomycin susceptibility test and KOH test. Our prospective study showed that out of 133 isolates, 99 (74.43%) were gram negative bacilli and 34 (25.56%) were gram positive cocci. Gram negative rods including *Escherichia coli*, *Klebsiella sp.*, *Pseudomonas sp.*, *Proteus sp.*, *Salmonella typhi*, *Citrobacter spp.*, *Enterobacter spp*. and gram positive cocci included *Staphylococcispecies (sp.), Streptococci sp. and Enteroccisp*. All gram negative bacilli shows 100% vancomycin resistance and KOH test positive.On the other hand, all gram positive cocci show 98.23% vancomycin sensitive, 1.76% resistance and KOH string test is 100% negative. Therefore, the alternative test of gram stain is Vancomycin susceptibility test and KOH test that are easily identify the gram negative and gram positive bacteria very well.
Keywords: Gram negative bacilli, gram positive cocci, vancomycin, KOH string test.

INTRODUCTION

The initial identification and classification of an unknown bacterium are largely dependent on the results of the Gram stain. Based on the gram staining reaction bacteria are classified into two groups i.e. the gram positive and gram negative bacteria where de-colorization is the major pitfall, as some gram positive bacteria decolorize more rapidly, and incorrectly identified as gram-negative[2-7]. Some factors, e.g., composition of the growth medium and the age of the culture [4] can influence the tendency of gram-positive bacteria to decolorize. The problem of gram-positive bacteria decolorizing is particularly evident with anaerobic bacteria; several strains characteristically stain gram negative or gram variable[8, 11].Several modifications of the Gram stain procedure have been developed to overcome this decolorization difficulty [3]. On the basis of their data, Stearn and Stearn concluded as follows:

- Gram positive organism can be rendered gram-negative by increasing acidity.
- Gram negative organism can be rendered gram positive by increasing alkalinecity.
- Basic stain-positive organisms can be rendered gram negative by increasing acidity.
- Acidic stain-negative organisms can be rendered gram positive by increasing alkalinecity.
- At the isoelectric range, there is little tendency for any stain to be retained. This range is characteristics of each species.
- There appears to be good evidence that the proteins of bacteria are not single proteins but a loose combination of proteins with lipoidal or fatty substances.
- The lipoidal material extracted from gram positive organisms differs from the extracted from gram negative organisms is thatthe former contains amuch larger proportion of unsaturated acids that have a greataffinity for oxidizing agents. All mordents used in the gram stain are oxidizing agents. Their effect is in general to sender the substances oxidized more acid in character. This increases that affinity of an organism for basic stains.
- The change of gram character with age is especially true of those organisms which are only weakly gram-positive and are cultivated

in media containing fermentable substances that become acid in reaction on growing procedure.

Many modifications techniques of the Gram stain procedure have been developed to overcome these decolourisation difficulties [11].

There have been several modifications of Gram's stain. These are:

Kopeloff and Beerman's modification

Primary stain solution consists of freshlyconstituted methyl violet with sodium bicarbonate in distilled water. Mordant consists of iodine dissolved in 4% NaOH solution. Decolorization is either using acetone alone or a mixture ofacetone and ethanol. Basic fuchsin is used to counter stain the smear. This method may be modified to stain tissue sections.

Jensen's modification

This method involves use to methyl violet as primary stain,iodine and potassium iodide in water as mordant, absolute alcohol as decolourizer and neutral red as counter stain. For Neisseria spp, Sandiford's counterstain is useful.

Weigert's modification

This modification is particularly useful for staining tissueSections. The primary stain carbol gentian violet is prepared using saturate alcoholic solution of gentian violet and 5% phenol solution. Gram's iodine is used as a mordant and aniline-xylol is used as a decolourizer. The counter stain carmalum (carminic acid and potassium alum in water), however is used ahead of primary stain. This method may be used to stain Pneumocystis cysts.

Preston and Morrell's modification

The primary stain used in this modification is ammonium oxalate-crystal violet. The smear is washed in Lugol's iodine and further treated with iodine solution. The smear is decolorized using iodine-acetone decolourizer and counterstained using dilute carbolfuchsin solution. This method has been further modified to overcome the irritating iodine in aerosols by reducing the iodine concentration to one-tenth and shortening the duration of decolorization to ten seconds.

Another method for the preliminary classification of bacteria is the use of a 3%solution of potassium hydroxide (KOH). Like the gram stain reaction, the KOH test is based on the differences in the chemistry of the bacterial cell wall. The cell wall of gram negative bacteria is easily disrupted when exposed to dilute alkali solutions [4].Another rapid method is the testing of susceptibility to vancomycin[9].

In the current study, Vancomycin susceptibility test and potassium hydroxide (KOH) test were used to differentiate bacterial isolates and these results were compared with standard gram staining.So many scientists to do work on the identification of pathogens by rapid test method but no one can do to identify the pathogens from Bankura area by this technique.

Present work highlights the rapid method todetermine the pathogenic-aerobic bacteria in different sources of samples without staining in the community of Bankura and surroundings, during March 2015 to June2015.

MATERIALS& METHODS

Sampling

A total of 133 community samples were collected from Bankura and surroundings, during March 2015 to June 2015.The samples were collected in 250 ml sterile containers and transported to the laboratory in cold conditions.

For isolation and identification

A total of 133 samples were isolated from the community of Bankura and surroundings, during March2015 to June2015. All samples were inoculated on a sterile MacConkey’s agar; Blood agar, Nutrient agar plates and the plates were incubated at 37°C for 18 to 24 hours. Plates were observed for growth. Then all the strains were subjected to gram staining, Vancomycin susceptibility test and KOH test.

Gram staining

Inthe first step, the bacterial smears were flooded with crystal violet for one minute and then washed gently in running tap water. In the second step, smears were exposed to Gram’s iodine for one minute, and then washed with tap water. In the third step, slides were exposed to acetone for decolourization and washed immediately with running tap water. Finally, dilute Carbol Fuchsin was added as the counter stain and washed after 60 seconds. After drying, stained slides were examined under oil immersion (100X) to note Gram reaction, morphology and arrangement[2, 4,10].

Vancomycin susceptibility test

Theisolates were tested by the modified Kirby-Bauer’s disk diffusion method on Mueller-Hinton agar plates. One-two colonies from the culture plates were inoculated into 2ml of peptone water and incubated at $37^{\circ C}$ for 2 hours. Turbidity was compared to that of 0.5 McFarland’s standard ($1.5x10^5$ CFU/ml). A cotton swab was immersed in this inoculums, the swab was then pressed to the sides of the tube so on the remove excess

inoculums. The swab was then used to inoculate the plate on the Mueller-Hinton agar in three different directions to ensure an even and complete distribution of the inoculums over the entire plat. The antibiotic disk was applied within 15 minutes of inoculation of plate. Vancomycin discs (5µg) were placed on the lawn culture and plates were incubated at 37^0C overnight. Any zone of inhibition was considered as sensitive[2, 4].

KOH-string test

Two drops of a 3% solution of potassium hydroxide were placed on a glass slide.Loop-full mass of bacterial culture, obtained from a 48-h culture on supplemented blood agar, was stirred in a circular motion in the KOH solution. The loop was occasionally raised 1 to 2 cm from the surface of the slide. The KOH solution characteristically became very viscous and mucoid with 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 s of mixing the bacteria in the KOH solution. Gram-positive bacteria suspended in the KOH solution generally displayed no reaction[2, 4].

OBSERVATION AND RESULTS

In our prospective study, we show out of 133 isolates 99 (74.43%) were gram negative bacilli and 34 (25.56%) were gram positive cocci. Gram negative rods including *Escherichia coli, Klebsiella sp., Pseudomonas sp., Proteus sp., Salmonella typhi, Citrobacter spp., Enterobacter spp.* and gram positive cocci included *Staphylococci species (sp.), Streptococci sp. and Enterocci sp.* shows in table-1. All gram negative bacilli show 100% vancomycin resistance and 100% positive KOH test. On the other hand, all gram positive cocci show 98.23% vancomysin sensitive, 11.76% resistance and KOH test is 100% negative. The results of the Vancomycin susceptibility and KOH test are given in table- 2.

Table 1: Distribution of Gram positive and Gram negative isolates

Gram Negative Bacilli	***No. Of Isolates***	***Gram Positive Cocci***	***No. Of Isolates***
Escherichia coli	72	*Staphylococcus spp.*	27
Klebsiella sp	11	*Streptococcus spp.*	5
Pseudomonas sp	8	*Enterococcus spp.*	2
Proteus sp	5		
Salmonella typhi	2		
Enterobacter spp	1		
TOTAL	**99**		**34**

Table 2: Results of the Vancomycin susceptibility and KOH test

Different isolates of bacteria	***Vancomycin susceptibility***		***KOH test***	
	Sensitive	Resistant	Positive	Negative
Gram negative bacilli	0	99(100%)	99 (100%)	0
Gram positive cocci	30(88.23%)	4(11.76%)	0	34(100%)

DISCUSSION

One of the essential and fundamental procedures is gram staining that identify the gram negative and gram positive bacteria. Present study highlights the alternative methods to identify the gram positive and negative bacteria by vancomycin sensitivity test and KOH string test. Gram negative bacterial cell wall dissolves with KOH 3 percent concentration whereas not in Gram positive cell walls. Dissolved cell wall releases the intercellular viscous or emulsified material out and it forms string. In our prospective study, we show that 99(100%) gram negative bacilli are vancomycin resistant and KOH positive. On the other hand 30 (88.23%) grams positive cocci are vancomycin sensitive and 4(11.76%) were resistance. Similarly, Arthi *et al*[1] studied that gram-positive bacteria showed 100% Vancomycin sensitive. Therefore, our result is a highly significant association between the results of Gram stain with KOH test and Vancomycin susceptibility.

CONCLUSION

The alternative test of gram stain is Vancomycin susceptibility test and KOH test that are easily identify the gram negative and positive bacteria very well.

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