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Full Length Research Paper

Virulence determination of bacterial isolates through culture in India ink including broth

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Virulency is the degree of pathogenicity exhibited by most of the pathogens and it is a measure that effectively differentiates pathogenic and non-pathogenic strains. In the present study, culture of bacteria for 10 to 12 h (overnight) at 37°C in 10% India ink included broth that can be used as marker test for initial virulence identification while screening a number of bacterial isolates. These results were further confirmed by the *in vivo* virulence tests. Although, this is the first report, there is no clear explanation to the degradation of India ink by virulent bacteria which was obtained but this could act as a marker for initial screening of isolates for their virulence. Moreover, India ink is used as a suitable capsular stain and capsule act as a virulence factor for most of the bacteria. So virulence test by 10% India ink including broth acts as a simple, low cost and easy method for initial screening of virulent from non virulent strains.

Key words: *Aeromonas hydrophila*, bacteria, India ink, pathogens, virulence.

INTRODUCTION

Virulence is the degree of pathogenicity exhibited by most of the pathogens and it is a measure that effectively differentiates pathogenic and non-pathogenic strains Russell and Herwald (2005). The degree of virulence depends on several virulence factors (Price, 2008) which includes capsular polysaccharide (Taylor and Roberts, 2005; Lindberg et al., 1979), capsule, DNase, coagulase, fibrinolysin, proteolytic, haemolysin, bacteriocin production, haemagglutination, serum sensitivity, epithelial cells attachment, hydrophobicity (Avila-Campos et al., 2000), lipase, antiphagocytic factor, biofilm (Prasad et al., 2009), extracellular enzymes production, presence of surface layer, lysine decarboxylase production and pili, (Pin et al., 1997) etc. There are several *in vivo* and *in vitro* assays to determine these virulence factors (Calderone et al., 2009). These include biochemical test (Voges-Praskauer reaction or fermentation of arabinose) (Kirov et al., 1998), tissue culture-based method (Yeung et al., 2007), tests using animal model (suckling mouse model) (Wong et al., 2006), molecular characterization by several virulence-associated genetic markers (Ma and Modi, 2002). All these methods though accurate are very tedious; need sophisticated laboratory and equipment, use live animals and time consuming. Some times it becomes difficult to handle quite number of isolates at a time.

Although, the *in vivo* or animal pathogenicity test has been taken for granted as the index for virulence for pretty long time, it is subjected to considerable variation because it reflects the interaction between host and the microorganisms, which is also affected by genetic and environmental factors, the relative susceptibility of the host. Moreover, it involves a lot of ethical issues and most of the countries also ban the use of live animals

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in research. To overcome this problem, the staining method is very useful and cost effective for initial screening of microorganisms as it determines the presence and absence of certain virulence determining characters such as presence of capsules by India ink (Fumiko et al., 2004), A- layer proteins (Udey, 1982), smooth -rough variations using coomassie brilliant blue (Cipriano and Bertolini, 1988) and also differentiate virulent from non-virulent by toluidin blue-iodine (Henry, 1920).

However, their accuracy depends upon various factors such as source of stain, staining methods, requirement of good microscope and visual interpretation. So in this study, we tested a more suitable method for initial screening of virulence from a number of isolates by culturing in India ink inclusion broth and it is the easier method to be practiced. So far, India ink has been used as capsular staining by several workers (Fumiko et al., 2004). Capsules are produced by all the virulent strains during infection but the capsule production and virulence are not correlated (Bennett et al., 2008). So in the present study, we evaluated the use of India ink in culture broth for their virulence.

MATERIALS AND METHODS

Several bacterial pathogens isolated from different diseases conditions of fish and human food products etc. were used. These include isolates of *Aeromonas hydrophila* (26)*, Flavobacterium branchiophilum* (3), *Flavobacterium columnare*(1), *Edwadsiella tarda* (6), *Pseudomonas fluorescens* (1), *Escherichia coli* (1)*, Streptococcus* sp. (4) and *Salmonella* sp. (1). These isolates were identified and properly characterized through standard microbiological methods (Cruickshank et al., 1975) and kept in slant at 4°C.

Different culture broths such as Brain Heart Infusion (BHI) Broth (Hi-media, India) or in Tryptic Soy Broth (TSB) (Hi-media, India) were prepared. India ink (Dye based Ink with CAMLI-SOL100) (Camlin Ltd., India) was filtered through 0.45 μm (Millipore, India)] and added to the broth at different concentrations (2.5, 5, 10, 15 and 20%). Then 24 h bacterial culture at 50 - 60 µl ml^{-1} was inoculated separately to each broth, incubated at 37°C and checked at definite time interval up to 48 h.

Selected strains were also then streaked on Coomassie brilliant blue (CBB) agar plate to check their virulence, as described by Cipriano and Bertolini (1988), simply adding CBB-R250 MS (s.d.Fine-chem.Ltd., India) to a final concentration of 0.01% in Tryptic soy agar medium and incubated in anaerobic condition for 24 h at 37°C.

In vivo virulence test were conducted using fish, *Labeo rohita* juveniles of average body weight ranging from 30 to 40 g. Fish were divided into several groups containing 12 numbers of fish in each group according to the number of selected bacteria taken for virulence study. The 24 h bacterial culture was centrifuged at 5000 xg for 10 min, washed in sterile saline and the final pellet was suspended in sterile PBS solution.

The fishes were injected intraperitoneally with 0.1 ml (10^6 - 10^9 CFU ml^{-1}) of bacterial suspension using three fishes per each dilution. Clinical signs and mortalities were recorded up to 7 days. Virulence was determined as the lowest dose, which caused 50% mortality, as calculated using the method of Reed and Muench (1938).

RESULTS AND DISCUSSION

During growth of these bacteria in India ink including culture broth, some isolates started degrading the ink after 5 to 6 h in only 10% India ink. However in 2.5, 5.0, 15 and 20% ink the degradation of ink color was not so quick, prominently visible and optimum at 10 to 12 h at 37°C unlike that of 10 India ink. Therefore, we have taken 10% India ink as our optimum and standard concentration for further virulence detection.

So keeping the culture in 10% India ink, the dye degradation was optimum at 10 to 12 h at 37°C (Figure 1A and B). By further increasing the culture time for more than 24 up to 48 h, all the isolates and strains degraded the color only in 10% ink. Those bacteria that degraded the color of the dye as early, within 12 h were checked for their growth on CBB agar plate, which showed the blue colonies (Figure 2B) while the other isolates which had no immediate effect on India ink grew as white colonies (Figure 2A). According to Cipriano and Bertolini (1988), CBB agar is a highly effective medium for the *in vitro* screening of virulence because this medium also helps in detection and quantification of the frequency of A-layer protein that acts as a major virulence factor. So this indicates that those bacteria that degraded the color of India ink were virulent unlike that of the other that retains the color as non-virulent. They were also further checked for their virulence by *in vivo* pathogenic trial in fish which showed the LD_{50} less than 10^7 CFU ml^{-1}. So this further confirmed that only the virulent bacteria could degrade the color of India ink in culture broth within 10-12 h which might be differenct in their biochemical and enzymatic processes. So, the present study showed that India ink including culture broth can be used as virulence marker and this method is easier, simpler in use, can be extended to other bacterial species and by this method we can primarily screen quite a number of bacteria at a time with low cost.

Though no clear explanation to the degradation of India ink by virulent bacteria has been obtained but this could act as a marker for initial screening of isolates for their virulence. Moreover, India ink is used as a suitable capsular stain and capsule act as a virulence factor for most of the bacteria. So the difference in culture behavior of bacterial isolates in presence of India ink may act as a marker for initial studying of bacteria, however they have to be further confirmed by different *in vivo* tests.

In conclusion, culture of bacteria for 10 to12 h (overnight) at 37°C in 10% India ink included broth that can be used as marker test for initial virulence identification while screening a number of bacterial isolates.

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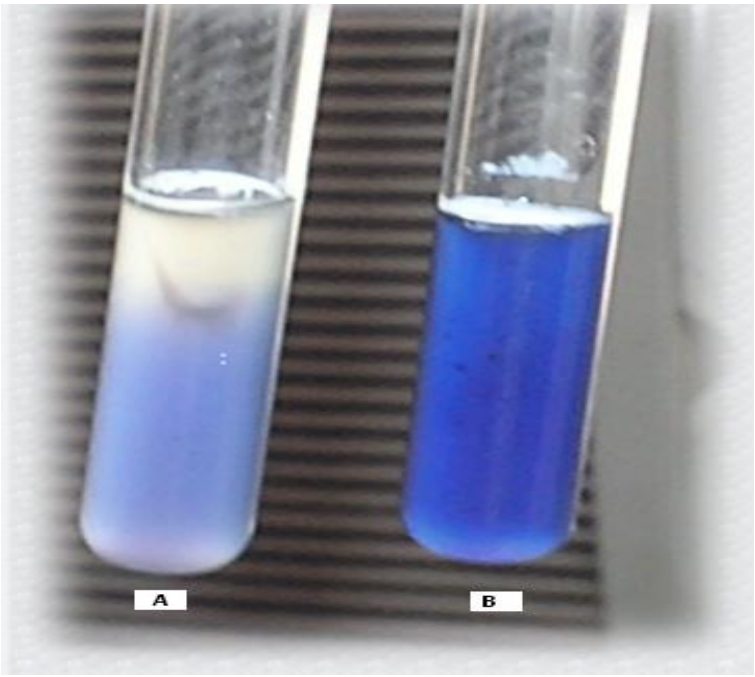

Figure 1. Growth of *Aeromonas hydrophila* in 10% India ink incorporated brain heart infusion broth; A: pathogenic strain, B: apathogenic strain.

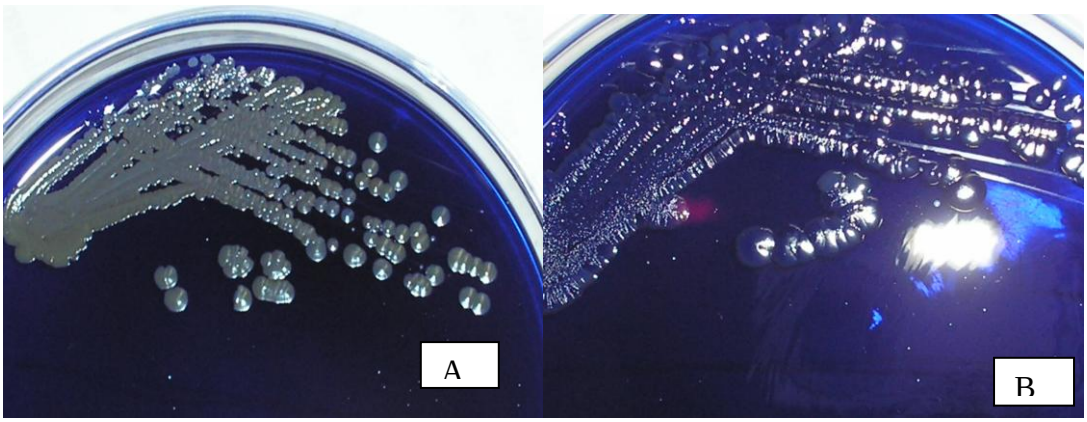

Figure 2. Growth of *A. hydrophila* in 10% India ink on coomassie brilliant blue (CBB) agar plate; A: apathogenic strain, B: pathogenic strain.

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