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Plant Heme: Future Approach for Characterizing Hemolytic Bacteria.

Abhishek Suman*, and Anjuvan Singh.

Department of Biotechnology and Biosciences, Lovely Professional University, Jalandhar-Delhi G.T. Road (NH-1), Phagwara 144411., Punjab, India.

ABSTRACT

It is quite common to use blood from various animal sources to characterize bacteria based on their hemolytic ability. Considering various drawbacks of using blood in culture, probably it's time to overlook for some proper potential alternative of blood that can serve the same purpose in a bacterial culture. In this regard, plant hemoproteins can be the one. These are found in various parts of plants such as root nodules of legumes in the form of leghemoglobin; leaves in form of cytochrome oxidase, peroxidases and catalases. The protoporphyrin ring present in these plant sources is similar to those present in animal hemoproteins. So, there is a room to use these plants derived heme-proteins as an alternative to blood and blood-derived products.

Keywords: 3R's, Plant heme-proteins, blood alternative, hemolysis.

Corresponding author

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INTRODUCTION

Heme, in the form of blood, is widely used as medium supplement in culturing many fastidious hemolytic bacteria such as *Staphylococcus aureus*, *Streptococcus pneumonia, Streptococcus pyogenes*, *Hemophilus* spp. and many others. Culturing such bacteria in the blood based medium serves various purposes in biological researches like immunological analysis, detection or diagnosis [1, 2], isolation [3, 4], characterization [5], growth [6, 7], checking hemolytic ability [8], drug discovery [9] and other related studies [10].

To meet the blood requirement, 15-20% of total blood volume of various cattle are harvested [11] on a large scale and the number is increasing annually. Various media have been formulated [12, 13], but they still require blood supplement.

Blood used in culture media are obtained from different sources like sheep, bovine, horse, and human is used in different cultures in amount 5-10% v/v [14, 15, 16]. Blood is mainly used for characterizing bacteria based on their hemolytic ability.

HEME-IRON UPTAKE MECHANISM

Numerous virulence determinants produced by bacterial pathogens gets activated in low-iron environments [17]. Hemolytic bacteria use these virulence factors to uptake iron from host heme proteins through various mechanisms. In a study on streptococcus pyogenes [18], it was described that various surface proteins of *S. pyogenes* binds to different hemoproteins such as hemoglobin, heme-albumin, myoglobin and hemoglobin-hepatoglobin and helps in iron extraction. Another hemolytic pathogen, *Vibrio cholerae* is believed to utilize more than one iron uptake mechanisms using hemolysins. The production of hemolysins in *V. cholerae* has been found to be an inverse function of iron, i.e. under iron starving condition, high amount of hemolysin production has been observed while in iron rich condition, its production gets decreased [19]. *Salmonella typhi* uses high affinity iron chelator enterochelin for acquiring iron from hemoproteins [20]. Similarly, *Pseudomonas aeruginosa* also secretes large amounts of two distinct siderophores, pyoverdin and pyochelin which act as strong iron chelators as well as transporters [21]. *Corynebacterium diphtheriae* produces an enzyme homologous to eukaryotic heme oxygenase which is used for extracting out iron component from heme [22]. According to a study done by [23], it has been known that some pathogenic *Escherichia coli* uses its specific ColV plasmid for iron acquisition from the host fluid. Other studies suggest the involvement of various transport proteins in the removal of iron from host compounds, such as transferrin, lactoferrin, heme, hemoglobin, and other heme containing proteins [24, 25].

HEMOLYSIS PATTERNS

Hemolytic bacteria are classified under three categories depending on the pattern of hemolysis they show in vitro. These patterns are:

Alpha (α) hemolysis – It is a partial or incomplete degradation of heme. In this, hemoglobin gets reduced to methemoglobin which appears as green-brown colored zone around the bacterial colony. Bacteria like *Streptococcus mutans*, *S. salivarius* show this characteristic.

Beta (β) hemolysis – It is complete degradation of heme. It is detected by clearing zone around the bacterial colony. Bacteria like *Staphylococcus aureus*, *Streptococcus pyogenes* show this characteristic.

Gamma (γ) hemolysis – It is non-degradation of heme. No change in color is observed around the bacterial colony. It also means that the bacteria is incapable of causing hemolysis. Bacteria like *Enterococcus faecalis* fall in γ-hemolytic category.

DISADVANTAGES OF BLOOD IN CULTURE

Due to desire, lack of proper potential alternative and lack of knowledge, blood is blindfolded used to serve earlier mentioned purposes. However, the use of blood in culture also bears a number of disadvantages:

Source of adventitious contaminants: Blood and blood derivatives are a major source of adventitious contaminants like prions, viruses, nanobacteria, Mycoplasma, etc. Since blood contains a large number of heat labile components, so it is almost impossible to sterilize it by autoclaving. Also, these contaminants are so small in size that they can't be separated by membrane filtration methods. These contaminants can easily affect the bacterial response as they share common host.

Batch variation: The composition of blood in different organisms varies with changes in individual [26] as well as environmental conditions, like day-hours, seasons, climate, etc [27, 28]. Thus, blood collected under different environmental condition results in different blood composition. In culture, blood is directly added as a supplement, so it has a synergic effect on the response of bacteria growing on it. Due to such variation, sometimes expected results are not achieved.

Difficult to sterilize: Blood, being highly complex and viscous, is very difficult to sterilize. If it is sterilized before supplying, it is not an easy task for the researcher to again sterilize prior to use. Thus, it also requires special attention in its safe storage.

Highly expensive: Blood or blood derivatives are highly expensive and costs up to 75% of the total culture cost.

Limited shelf-life: Blood, blood derivatives and blood-based media have very short and limited shelf life. Post-expiry use of such products in medium may lead to erroneous result.

High demand to supply ratio: Continuously increasing research on hemolytic bacteria has exponentially increased the demand of blood medium, but the production and supply is limited. To increase in supply, more animals need to be harvested.

Safety-, regulatory- and ethical- issues: Blood is an animal derived product, so its collection is also a matter of ethical concern. If a large amount of blood is drawn frequently without replacement, animals may develop hyperpnea and experience hypovolemic shock [29]. Long term excessive bleeding causes a number of diseases in animals, including reduced exercise tolerance and ill thrift [30]. Blood is not produced in every country, rather it is supplied. The supply depends on the political relationship between the provider and recipient countries. This leads to regulatory issues. Again, use of blood is sometimes not safe to use because the person involved in experimentation may get infected by coming in contact with the blood.

Cause damage to the environment: The cultures are generally discarded after experimentation. On exposure to the environment, due to their high nutritional content, they attract a large and diverse family of micro-organisms, allow their rapid growth and cause damage to the environment.

Source of development of MDR (Multi-Drug Resistant) strains: If the blood-donor had ever undergone any type of drug treatment and its blood is used in the medium, it may be a potential source of MDR strains of various microbes.

Incompatibility of blood with bacterial growth: Blood from any source can't be supplemented to the basal medium for detecting hemolytic activity [31].

3R'S PRINCIPLE

Considering the disadvantages of supplementing blood in basal medium for hemolytic bacteria, it becomes necessary to promote the 3R's principle given by Russell and Bruch [32, 33]. This principle describes in detail the possible alternatives to animal experimentation:

Replacement – This is the alternative of first priority to replace the animal with any other possible choice including plants.

Reduction – If a replacement is not possible, then second alternative can be to reduce the number of animals used in experimentation.

Refinement – Being the last alternative, it focuses on comprehend those methods that alleviate potential pain and distress; and enhance animal health.

It seems quite paradoxical to use blood from animal sources in *in vitro* studies in favor of 3R's principle.

HEME FROM PLANT SOURCES

In plants, heme is present in a number of proteins, like chlorophyll, cytochrome c oxidase, peroxidase, catalase and plant hemoglobins [34]. However, not all heme in plants has iron (Fe^{2+}) center. Like in chlorophyll, instead of Fe2+, Mg2+ serves as the central metal ion and so it is called as magnesium porphyrin [35]. Apart from chlorophyll, many other hemoproteins contain an iron prophyrin ring in their core and are similar to that present in animal system [36, 37, 38]. Hemoglobins in the plant was first identified in Soybean root nodules and further in a number of legume plants. In plants, hemoglobin is present in various parts such as root nodules and plastids in leaves [39, 40]. Based on their sequences, expression patterns, and ligand-binding properties, plant hemoglobins are classified under two major categories:

Symbiotic hemoglobin - These are mainly found in the infected cells of nitrogen-fixing nodules of legumes and non-legumes. Their main function is to facilitate oxygen transport.

Non-symbiotic hemoglobin - These appear to be ancestral to the symbiotic Hemoglobins. These are widely spread throughout the plant kingdom and found in various types of plant tissues. Generally, they display high affinity for oxygen molecule.

PLANT VS. ANIMAL HEME

The main reason of considering heme-proteins obtained from plant sources, for bacterial culture instead of blood, is that the iron-porphyrin ring present in plant heme proteins are similar to that present in animals [36]. Structural comparison of heme from 3 different plants heme-proteins and 1 human heme-protein, using Bioinformatics tool, showed insignificant variation with respect to human fetal hemoglobin.

WHY TO USE PLANT HEME-PROTEINS?

Using plant derivatives as a constituent and supplement in culture media has its own perks. Most importantly, there are no ethical and regulatory issues related to use of plant products. It is also safe to use such materials in culture due to low risk of indigenous contamination. If discarded, then also they get degraded very easily and do not cause any adverse effect on the surroundings, thus it is also eco-friendly. Furthermore, plant resources are present in abundance and can be easily cultivated whenever required. Since, greater amount of products can be obtained by consuming less plant resources, it is possible to meet supply according to demand. Being user-friendly, easy to prepare such supplements even at lab scale, it will also minimize the culture cost. Another important aspect of using such products is that they can be easily sterilized by filtration techniques.

FUTURE APPLICATIONS

The plant heme-proteins may have the potential to serve all the purposes mentioned earlier. It can be used as effective alternative technique for detecting hemolytic activity as well as a tool for bacterial characterization. Being cost-effective, it can be used in routine culture and maintenance of hemolytic bacteria. Being a medium supplement, it can allow researchers using this in their various heme related researches instead of blood or blood derivatives.

CONCLUSION

Based on all these discussions, we hypothesize the potentiality of plant heme-proteins to be the possible alternative of blood leading to the development of entirely new approach of characterizing hemolytic bacteria.

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