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ORIGINAL RESEARCH

Qualitative Blood Plasma Moderation of in Vitro Staphylococcal Catalase Activity and its Implications for Invasive Bacterial Infections

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Abstract:

Introduction: Staphylococci are clinically-important bacteria notable for catalase production when grown *in vitro* and during host infections. However, the comparative activities of the enzyme in the presence and absence of different chemical components in body fluids are apparently unknown.

Aims: This study investigated the qualitative influence of blood plasma on *in vitro* activity of staphylococcal catalase and virulence potentials of the enzyme-producing organisms, *Staphylococcus* strains.

Materials and Methods: Twenty-seven (27) biochemically identified clinical *Staphylococcus* isolates, comprising 33% coagulase positive strains, were suspended in 2 mL sterile normal saline and screened for catalase production by tube and slide techniques, using 6% (w/v) hydrogen peroxide (H_2O_2). The characteristic bubbling reactions and duration were compared with separate volumes of the same cell suspensions mixed with equal amounts of some randomly selected human blood plasma. The reactions of blood plasma samples treated with H_2O_2 served as another control.

Results: All the bacterial suspensions, free of blood plasma, showed immediate vigorous bursting bubbling reaction for a few seconds but those mixed with plasma took about 15 seconds before producing tiny bubbles and progressed to forming profuse foams, rising high in narrow tubes or extending beyond the droplet spots. The bubbles generated stayed for several minutes without busting. Similarly, the control blood plasma- H_2O_2 interactions slowly yielded persisting bubbles but comparatively much smaller in quantity.

Conclusion: This study revealed blood plasma has the potential of slowing down the characteristic intense rapid *in vitro* activity of staphylococcal catalase activity and such suboptimal enzyme performance may reduce oxidative stress survival of the bacteria during invasive infections.

Keywords: *Staphylococcus*, catalase, hydrogen peroxide, reactive oxygen species, bacteria, infection, oxidative stress.

All co-authors agreed to have their names listed as authors.

1. **INTRODUCTION**

Staphylococci are facultative, non-motile, spherical-shaped, Gram-positive bacteria with clustered cell arrangements and can withstand high salt concentrations of 7.5-10% (w/v). They are commonly found on the skin and mucous membranes of the respiratory tract of warm-blooded animals as commensals, but sometimes cause diseases of varying severity in their hosts [1]. Thus, staphylococci are designated as opportunistic pathogens and most often infection results from breaches in the host's skin or mucous membrane barriers or contaminated catheters. The most frequently isolated member of the genus from clinical samples is *Staphylococcus aureus* and it causes skin infections, such as boils, folliculitis and sometimes pneumonia, sepsis, bacteremia, endocarditis, and osteomyelitis. Some strains produce toxins that cause food poisoning, scalded skin syndrome, and toxic shock syndrome [2-3].

The genus *Staphylococcus* consists of about forty species and its members are generally classified into two main groups: coagulase-positive staphylococci (CoPS) and Coagulase-Negative staphylococci (CoNS) [3]. Though the number of species in the genus is over 35; only about seven of them are coagulase positive and commonly represented by *S. aureus*, which produces many tissue degrading enzymes like fibrokinase, DNAse and some strains elicit toxins among other characteristic virulence factors. Coagulase-negative staphylococci (CoNS), on the other hand, are typified by *S*. *epidermidis* and *S*. *saprophyticus* and have limited known virulence factors [2]. The spectrum of human diseases due to these opportunistic pathogens ranges from occasional self-limiting pimples, and boils to systemic infections requiring therapeutic antibiotic interventions [4].

Staphylococci, like other oxygen-dependent organisms, may generate some toxic oxygen derivatives as by-products of normal metabolism, which encompass singlet oxygen ($^1O^{2-}$), superoxide free radicals ($O^{2.-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH•$) [5]. These reactive oxygen species (ROS) are associated with the potential destruction of cellular proteins, enzymes, nucleic acids, and other cellular components [6-7]. In order to mitigate the cellular toxicity of the aforementioned, affected organisms produce enzymes, such as superoxide dismutase, glutathione peroxidase, and catalase among other known ROS detoxification mechanisms [8]. However, hydrogen peroxide is also produced by macrophages as the first line of the internal body defense system against infections and the cell secretes hydrogen peroxide to provide an effective chemical weapon against many infectious agents [9-11]. Thus, staphylococcal catalase functions to provide relative protection for the producing organisms both *in vitro* when cultured on artificial media in the laboratory, on the host skin, and *in vitro* during infections of susceptible hosts.

In vitro studies reported catalase as an enzyme with the highest turnover numbers compared to all other enzymes. One molecule of this enzyme catalysis rapid decomposition of millions of hydrogen peroxide molecules to water and oxygen per second, ($2H_2O_2 \rightarrow 2H_2O + O_2$) [12]. However, during invasive infections, the bacterial enzyme is exposed to a myriad of dissolved organic and inorganic chemicals in body fluids, notably the blood plasma or its derivatives, the lymphs. Though enzymes are generally specific in the chemical reactions they catalyze, their activities may become altered in the presence of extraneous chemical substances capable of modifying the enzymes' molecular structures or competing with substrates for their active sites [2]. The resultant effect of the interactions of some of these substances with target enzymes is either an increase or decrease in rates of transformation of specific substrates to their products [13].

However, if an infectious agent must survive and multiply in the presence of activated host defense, including the production of hydrogen peroxide, it requires, among others, optimal and rapid catalase neutralization of the host's hydrogen peroxide. Hence, this study simulated an *in vivo* condition of exposure of the bacterial catalase to typical composites of human body chemical substances during tissue/invasive infection, qualitatively compared the enzyme activities with its classic *in vitro* activity and its consequences for the bacterial virulence potentials.

2. MATERIAL AND METHODS

2.1 Collection and Description of Test Bacterial Isolates

Thirty (30) clinical bacterial isolates, which had been tentatively identified on the basis of growth on selective media and microscopic characteristics as staphylococcal strains were obtained from the Department of Microbiology, Faculty of Science, Lagos State University, Ojo. The isolates were stocked on nutrient agar (NA) slants and maintained in the laboratory by refrigeration. The test organisms were sub-cultured and purified, Gram-stained and subjected to biochemical tests for identification [14].

2.2. Purification and Characterization of Isolates

The bacterial isolates were sub-cultured and purified on sterile nutrient agar (OXOID) plates under aerobic incubation at 35-37 ^{o}C for 18 - 24 h. Slide smear preparations of actively growing pure cultures were Gram-stained and examined. Salt tolerance and mannitol fermentation characteristic of isolates was determined by growing them on Mannitol Salt Agar (MSA), which is a selective medium for the differentiation of pigment-forming *Staphylococcus* species. Other biochemical identification tests performed include the detection of the presence of cell wall-associated Protein A (agglutinins), production of extracellular blood plasma coagulating enzymes (coagulase) and nucleic acid hydrolytic enzymes (DNAse). These tests were carried out as described by Arora and Arora [1].

2.3. Determination of Catalase Production by Test Organisms

Colonies of the test organisms were picked from Nutrient agar plates with a non-hydrogen peroxide reactive wire loop and were transferred into droplets of hydrogen peroxide (6%, w/v) inside sterile Petri dishes or 0.5 mL of the peroxide contained in test tubes. In the modified test procedure, the test organisms were suspended in 1 mL sterile normal saline, ensuring the resultant cell suspensions were highly turbid with general cloudiness of about 0.7 McFarland Standard ($2.7x10^{9}$ CFU/mL). Instant bubbling reactions of the organisms, on exposure to hydrogen peroxide were considered positive catalase activity. Laboratory stock *Enterococcus faecalis* strains served as catalase-negative test control [15].

2.4 Screening of Plasma Samples for Catalase Activity

The plasma samples used in this study were aspirated from ten (10) randomly selected blood samples in 5 mL EDTA plastic containers, which had been used for some routine laboratory analyses at the Lagos State University's Health Centre and kept for disposal. The plasma samples selected were free of any observable evidence of red blood cell lysis and contamination. They were tested for catalase activity by placing two (2 drops of each sample inside sterile Petri dishes and equal amounts of hydrogen peroxide (6%, w/v), (2 drops) added to them. The mixtures were observed for any bubbling reaction as an indicator of positive catalase activity.

2.5 Effects of Blood Plasma on Bacterial Catalase Activity

The earlier described thick bacterial cell suspensions and plasma samples that produced little or scanty bubbles on reacting with hydrogen peroxide were used here. Twenty-five microliters (25 uL) of the bacterial suspensions were dropped at different spots on two (2) sets of Petri dishes, labelled A and B Thereafter, equal amounts of the plasma (25 µL) alone were dropped in Petri dishes C and to different bacterial suspension droplets in Petri dishes B while sterile normal saline in the above quantity was added the bacterial droplets in Petri dishes A. Similar cell /plasma preparations of 50 µL were repeated in test tubes A, B and C as against 25 µL of the reactant droplets in Petri dishes. Thereafter, the same amount of hydrogen peroxide, 25 µL added to all the droplets on Petri dishes and 50 µL to those in test tubes. The bubbling characteristics of the bacterial suspensions mixed with plasma samples in dishes/tubes B were compared with plasma-free cell suspensions in dishes/tubes A and cell-free test plasma C.

3. RESULTS

Twenty-seven (27) (90%) of the thirty (30) presumptively identified clinical staphylococcal bacterial isolates were grown on the MSA selective medium and twelve 12 (44.4%) among them formed golden yellow colonies. Microscopic examinations showed they were Gram-positive with round-shaped cells arranged in clusters. Coagulase and DNAse production were detected only among nine (9) members of the yellow colonies forming isolates, identified simply as coagulase positive (33.3%) and others as coagulase-negative *Staphylococcus* strains. The reactions of hydrogen peroxide directly with the bacterial colonies or cell suspensions without plasma were characterized by instant, vigorous, bursting bubbles that generally lasted for a few seconds. About 70% of the ten (10) test blood plasma samples exhibited varying low amounts of catalase activities, while 30% formed no bubbles on exposure to hydrogen peroxide. All the catalase reactive plasma samples slowly formed non-bursting bubbles, aggregated as small persistent foams (Fig. 1).

Figure 1: Variability in catalase activities of screened blood plasma samples based on bubble formation on reaction with hydrogen peroxide.

However, when the bacterial suspensions, mixed with equal volumes of blood plasma, were treated with test hydrogen peroxide, there was a time lag in the observed bubbling response and with distinct forming characteristics. The enzyme activity manifested in poor bubble formation, which gradually progressed to a profuse foaming reaction. Secondly, the bubbles were observed to be tiny and aggregated in forming foams, rising up in tubes, and in Petri dishes/slides, the foams covered and extended beyond the spots of the reactant droplets. Thirdly, the profuse foams persisted (Fig. 2 and Fig. 3) for several minutes, sometimes lasting 30 minutes or beyond before any appreciable bursting and reduction in foam quantity were noted.

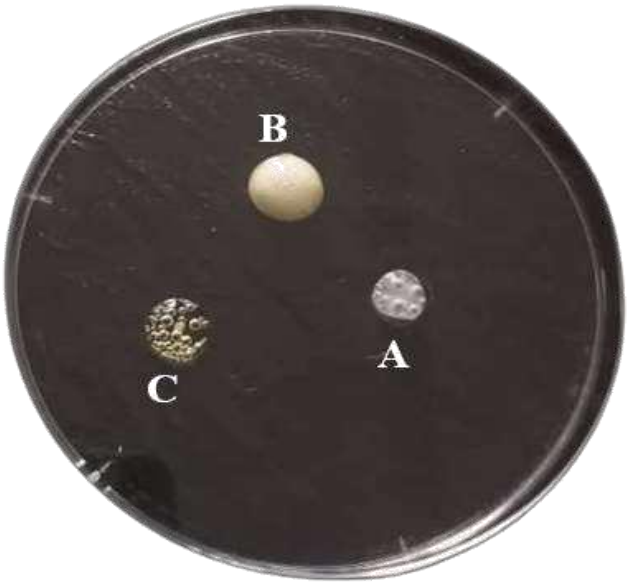

Figure 2: Comparative catalase activity of 25 µL *Staphylococcus* cell suspensions after about five minutes of reaction time. A: Cell suspension alone, B: Cell suspension mixed with blood plasma, C: Control test plasma.

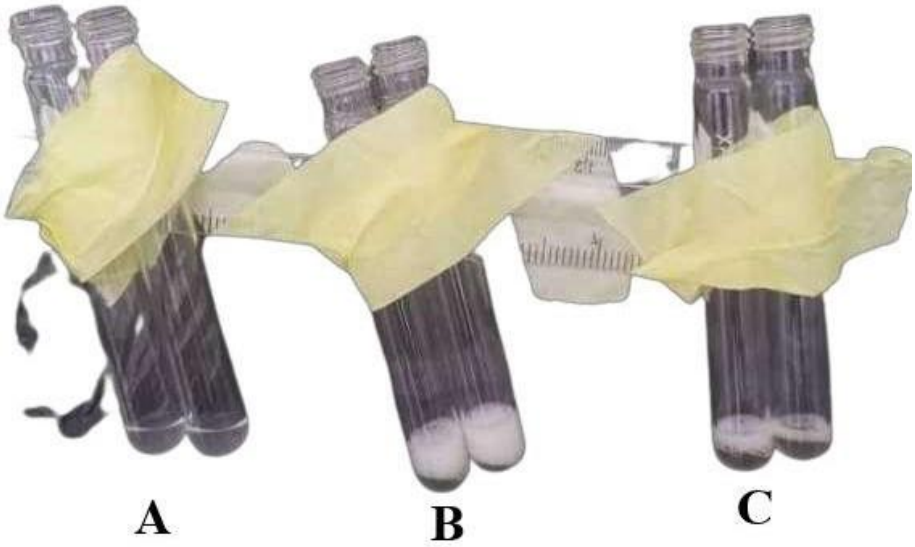

Figure 3: Comparative catalase activity of *Staphylococcus* cell suspensions in tubes, after about five minutes of reaction time. A: Cell suspension alone, B: Cell suspension mixed with blood plasma, C: Control test plasma.

4. DISCUSSION

The successful growth of the test bacterial isolates on Mannitol Salt Agar (MSA) when considered together with their colonial appearances, cell morphology and biochemical reactions are consistent with phenotypic characteristics of *Staphylococcus* species [14-15]. Indicators of virulence potentials (agglutinins, coagulase, and DNAse production) exhibited by 33% of the organisms are typical of the classic pathotypes, designated as (CoPS), particularly *Staphylococcus aureus* [16]. The observed differences in the number of this pathotype strains and (CoNS, 66%) among the randomly collected clinical strains of the bacterial used for this study are probably due to the natural relative dominance of CoNS members in the genus, particularly *S. epidermidis, S. saprophyticus* among others [16].

In this study, both the conventional and modified test procedures showed *Staphylococcal* strains as strong catalase-producing bacterial organisms. Though these two methods are quite simple to perform, the conventional method, commonly used for routine laboratory detection of catalase, is undoubtedly faster than the latter as it does not involve any pretreatment of the required bacterial colonies [2]. However, the modified technique lends itself to both qualitative and quantitative analytic procedures as the test bacterial load (CFU/mL) in suspension can be determined by different protocols [13], varying the turbidity of cell preparations required in determining the outcomes of their biochemical activities.

The observed vigorous, bursting bubbling catalase reactions of bacterial isolates in absence of any pre-treatment with blood plasma sample is evidence of high activities of the enzyme as reported by Matzov *et al.* [12]. The time lag of several seconds before the cell suspensions mixed with blood plasma showed some bubbles, though quite poor (tiny sizes), before progressing to the steady formation of foams (Fig 2) suggesting the presence of blood plasma exhibited some influence on the enzyme-substrate interactions. Foam is an aggregation of bubbles and because the bubbles of the later reaction persisted as unbusted foams for several minutes indicated the blood plasma ad-mixed catalase reaction was less intense [14]. In other words, it appears enzymatic breakdown of the peroxide with concomitant release of molecular oxygen as bubbles became reduced but progressed steadily for a longer period in contrast with the nature of the action of the blood plasma-free cellular reactions.

Though the underlying mechanism(s) of the blood plasma's action on the staphylococcal catalase activity is not known for now since its involvement caused the reaction to become less extreme, or non-vigorous, its influence can be regarded at present as a potential moderator or regulator in the enzyme-substrate interactions [17]. Again, the lag phase of a few seconds required for the bubbles to begin to appear might be the period required by the blood plasma components to interface with the enzyme-substrate interaction in preparation for the moderation of the enzyme's activity manifested in the observed steady foaming reactions.

Meanwhile, the varying amounts of foaming reactions exhibited by some of the blood plasma droplets treated with hydrogen peroxide as control (Fig. 1) may be due to the presence of variable amounts of catalase in body tissues [18-19] and or substances like iron derivable from lysed red blood cells and active on H_2O_2 [20-21]. The similarity in foaming reaction shown by the test plasma samples and plasma ad-mixed bacterial preparations (Fig. 3) suggests the earlier suspected catalase moderating substance in plasma may be naturally present in the blood to regulate activities of catalase by the body, either generated by a host or extraneous agents within the host, invasive pathogens inclusive. The commonly observed *in vitro* vigorous reactions may be too high and unsafe for body tissues, thus the catalase activity moderating agents are required in animals to function in a manner similar to systematic enzyme release of energy from a catabolite [2,13].

Finally, considering the complex intimate interactions between a disease causative agent and susceptible hosts during infections, the presumed host tissue protective benefits of moderation of *in vitro* catalase activity observed in this study may be detrimental to the survival of invasive pathogens in infected hosts [22]. These causal agents naturally need to rapidly neutralize the hydrogen peroxide formed by the hosts' immune system, if it must survive and multiply, and a reduction in the bacterial catalase activity, which is synonymous with suboptimal performance of the enzyme capable of compromising or reducing the pathogens' ability to effectively counter the antimicrobial oxidative stress [23]. This may significantly

contribute to the eventual immunologic destruction and elimination of the pathogen, perhaps in most subclinical infections.

5. CONCLUSION

This study shows the characteristic *in vitro* vigorous activity of staphylococcal catalase can be moderated by blood plasma and such suboptimal performance of the enzyme may reduce the ability of the bacteria to survive host tissue-elicited oxidative stress during invasive infections

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COMPETING INTERESTS

The authors declared that no competing interests exist

AUTHORS' CONTRIBUTIONS

KA designed the study and performed the protocol with SA. CF wrote the first draft of the manuscript while OL managed the literature searches. All authors read and approved the final manuscript.

CONSENT (WHERE EVER APPLICABLE)

Not applicable

ETHICAL APPROVAL (WHERE EVER APPLICABLE)

No human subjects were involved in the study.

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