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Evaluation of Bactericidal Activity of Various Human Serum Group against *Staphylococcus aureus*

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Abstract
The effect of various human serum groups bactericidal activity was evaluated in vitro against S. aureus using both complement and non-complement pathways. Human serum contains a group of more than 30 proteins collectively known as complement systems that facilitate phagocytosis, cell wall lysis and other protective means against pathogens. Isolates of S. aureus were recovered from wound samples, cultured and identified using conventional Gram stain, biochemical methods and molecular means. A total of147 human were harvested from vein punctured blood and typed by reverse blood grouping method, Samples were also categorized into children, adults and elder's serum. Serum bactericidal assay was measured by inoculating 0.25ml of S. aureus suspension in equal amount of fresh serum, EDTA treated serum and heat inactivated serum followed by incubation at 37^{O}C for 60 minutes. Finding of this study shows that S. aureus is less sensitive to ABsera group(66.65%) compared to A, B and O serum groups (68.97%), (69.91%) and (69.73%) kills percentage respectively for fresh serum while EDTA treated revealed an average killing percentage of 57.39%, 57.09%, 59.38% and 59.92% kills for "A", "AB", "B" and "O" sero-grouping respectively. Also the study revealed that adult sera indicated a significant difference in S. aureus killing compared to children and elderly ($p \leq 0.05$). It was found that human serum bactericidal activity increases with age until a certain level (45 years) that the activity seems to decline. This study recommend that new therapeutics target should concentrate on the bacterial modulators for immune proteins.

Keywords: Bactericidal activity, Complement, Evaluation, Human serum, *Staphylococcus aureus*.

Introduction
An estimated 20% to 30% of the human population are long-term carriers of *S. aureus* which can be found as part of the normal skin flora, in the nostrils, and as a normal inhabitant of the lower reproductive tract of women (Baddam *et al.*, 2012). *S. aureus* can cause a range of illnesses, from minor skin infections, such as pimples, impetigo, boils, cellulitis, folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases such as

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pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia, and sepsis(Meyer *et al.,* 2014). It is still one of the five most common causes of hospital-acquired infections and is often the cause of wound infections following surgery (Rochelle *et al.,* 2007). The bactericidal effect of serum is an essential innate immune mechanism of the human host that provides protection against harmful bacteria (Yehoshua *et al.,* 2014). The protective capacity of antibody and complement proteins in the serum is referred to as complement-mediated bactericidal activity or serum bactericidal activity and is determined via an *In-vitro* technique known as serum bactericidal assay (SBA) (Mcintosh *et al.,* 2015).

The serum bactericidal test (SBT) is a modification of the broth dilution method that measures the bactericidal activity of the patient's serum during antimicrobial therapy against the bacterial pathogen isolated from that patient (Zhou, 2012). It is one of the few in vitro tests performed in the clinical Microbiology laboratory that incorporates the interaction of the pathogen, the antimicrobial agent, and the patient (Gorsuch *et al.,* 2012).

Several studies have investigated the bactericidal activity of serum against different pathogens. It is widely known that the *In-vitro* killing of bacteria by serum is mainly complement-driven (O'shaughnessy *et al.,* 2012). In this study the bactericidal activity of various human serum groups against *Staphylococcus aureus* wound isolates was evaluated, in this regard individual serum were test rather than the conventional pooling method also complement activity was inhibited to perfectly determine its level of activity.

Materials and Methods

Ethical Consideration

Study was conducted with an ethical clearance of the Kano state Ministry of Health for the period of five months (March – July, 2020).

Study Area, Sample Collection, Serum Processing and Harvesting

Total of one hundred and forty seven (147) human serum were collected from asymptomatic volunteers within Wudil town by venipuncture, their sero groups were determined by reverse blood grouping method using laboratory prepared anti-A and B cells in Microbiology unit, laboratory department of Wudil general hospital, Kano, Nigeria as described by NFL, (2012) and Dhurba, (2017).

Staphylococcus aureus Strain Enumeration

Strains of *S. aureus* were recovered from wound swaps by culturing the sample on Mannitol Salt Agar at 37^{O}C for 24 hours, colonies were confirmed by Gram's reaction and other convectional biochemical methods described by Cheesbrough (2012) and American Public Health Association (2018). Molecular identification was done in the multipurpose laboratory of Ahmadu Bello University, Zaria in Kaduna, Nigeria. Genomic DNA extraction was based on the method described by Norgen Biotech (CANADA). Extracted DNA samples were quantified on agarose gel electrophoresis to determine DNA size and assess the RNA contamination.

Polymerase chain reaction amplification was carried out using-storm thermal cycler. Products of the amplification were electrophoresed and visualized under UV illumination in Gel Documentation system 2000 (Biorad, Hercules CA, USA) and stored as TIFF file format. Sizes of the amplicons were estimated in comparison with 50bp DNA ladder (CLEAVER SCIENTIFIC LTD, UK). A1.5kb band of DNA was excised from gel and purified using gel elution kit (Sigma-Aldrich, USA) based on the manufacturer's protocol. Sequencing

reactions were carried out with a Big Dye Terminator cycle sequencing kit (Applied Biosystems, USA), standard universal primer forward (8f ') and reverse (1542r') primer and sequenced by using ABI Prism 3100 genetic analyzer (Applied Biosystems, USA).

The sequences thus obtained were assembled and edited using Clone Man- ager Version 5 (http://www.scied.com/pr_cmbas.htm). Database search was carried out for similar nucleotide sequences with the BLAST search of Non-reductant (NR) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Partial length 16S rRNA gene sequences of strains closely related to the isolate were retrieved from National Center of Biotechnology Information (NCBI) for further analysis.

Serum Bactericidal Assay

From the harvested serum 0.25ml were pipetted and transferred into a three sterilized test tubes, to the first tube serum was subjected to heat inactivation at 56^{O}C for 30minutes in a rotary water bath, to the second tube 0.25ml of EDTA was also transferred, third tube contains the fresh serum only, each followed by an equal amount (0.25ml) of the bacterial suspension, the whole mixture was then incubated in water bath (Wincom Company ltd.; Model DK-420) at 37^{O}C for 1 hour, after which the mixtures were poured to separate sterilized Petri dish followed by 15ml of molten Nutrients Agar (NA), allowed to solidify, inverted and aerobically incubated at 37^{O}C in an incubation machine (Techmel and Techmel USA; Model TT-9052) for 24 hours. Colony formed were read and express in colony forming unit per milliliter (cfu/ml) (Owhe-Ureghe and Okoh, 2008). The same was done to all the samples processed.

Bacterial species were considered sensitive, if more than 50% killing was obtained by modifying the formula described by Microchem Laboratory (2015):

$$Kill\ (\%) = \frac{No.of\ cfu\ mL^{-1}\ H - No.\ of\ cfu\ mL^{-1} F}{No.of\ cfu\ mL^{-1}\ H} x\ \frac{100}{1} \qquad \text{Equation (1)}$$

$$Kill\ (\%) = \frac{No.of\ cfu\ mL^{-1}\ H - No.\ of\ cfu\ mL^{-1} E}{No.of\ cfu\ mL^{-1}\ H} x\ \frac{100}{1} \qquad \text{Equation (2)}$$

where *H* is the Heat inactivated serum bactericidal activity, *F* is Fresh serum bactericidal activity and *E* is EDTA treated serum bactericidal activity

Data obtained was subjected to two-way analysis of variance and considered significant at $p \leq 0.05$.

Results

This study showed the serum bactericidal activity of three categories of human serum against isolates of *Staphylococcus aureus* with respect to four different sera groups of the participants. Three types of sera were examined for their activities against the isolates, the fresh human serum, the Ethylene-Diamine-Tetra-Acetic acid (EDTA) treated serum which inactivated the phase ways of complement and the heat inactivated serum which served as the negative control.

The result showed that, sero-group AB has the highest participants of 52(35.37%) followed by B sero-group with 37(25.16%) participants. However, least count of participants were found from both A and O sero-groups with 29(19.73%) participants each (Table 1).

Moreover, the complement mediated susceptibility testing shows a promising bactericidal activity set at 50% kill of isolates, 28(96.55%) participants with "A" sero-grouping shows an average killing percentage of 68.97%, 49(94.23%) participants with "AB" sero-grouping

showing an average killing percentage of 66.65%, 36(97.29%) participants with "B" sero-grouping shows an average killing percentage of 69.91% while 29(100%) participants with "O" sero-grouping shows an average killing percentage of 69.73% (Table 1).

Furthermore, the non-complement mediated susceptibility testing shows a compromising bactericidal activity compared to complement driven activity set at 50% kills of isolates, 16(55.17%) participants with "A" sero-grouping shows an average killing percentage of 57.39%, 35(67.31%) participants with "AB" sero-grouping shows an average killing percentage of 57.09%, 24(82.76%) participants with "B" sero-grouping shows an average killing percentage of 59.92% while 20(68.96%) participants with "O" sero-grouping shows an average killing percentage of 59.92% (Table 1).
The bactericidal activity of fresh children serum shows an increasing activity with addition of age thus, 2 years age serum kills 48.80% of the isolates while 12 years age serum kills 65.50% of the isolates at 60 minutes of inoculation. However, the EDTA treated child serum shows an absolute compromise activity where by 2 years age serum kills 40.19% of the isolates while 12 years age serum kills 48.84% of the isolates as shown in figure 1.However, both fresh and EDTA treated sera showed no significant difference with regard to the child participants' sero-groupings at $p \leq 0.05$.

Moreover, fresh adult's serum shows a more auspicious bactericidal activity as shown in figure 2 in which 13 years age serum killed 68.71% of the *S. aureus* isolates while the 40 years serum kills 78.03% of the isolates at 60 minutes after inoculation. On the other hand, the EDTA treated adult serum showed an irregular acceleration and retardation at the same time from 13 years of age to 40 years though, there is a relatively increase. A 13 years age serum kills 56.12% of isolates while that of the 40 years age kill 53.18% of the *S. aureus* isolates. Yet, both fresh and EDTA treated sera shows no significant difference with regard to the adult participants' sero-groupings at $p \leq 0.05$.

Furthermore, fresh elderly serum like adults' serum shows a fortunate activity with the addition of age though decelerate from 41 years which kills 85.82% of isolates and on reaching 58 years old, the activities decelerate to 69.96%. Conversely, the EDTA treated serum of elderly participants shows also an irregular curve. Though, the activity decelerate with the addition of age where by 41 years age serum kills 68.97% while 58 years age serum kills 57.14% of the isolates. Still, both fresh and EDTA treated sera shows no significant difference with regard to the elderly participants' sero-groupings at $p \leq 0.05$.

Table 1: Serum Bactericidal Activity based on ABO Sero-grouping of the Participants against the Isolates

Serum Group	Subject Categories			Total (%)	Bactericidal Activity	
	Child (%)	Adults (%)	Elders (%)		CD (A %)	CI (A %)
A	13(8.84)	11(7.48)	5(3.40)	29(19.73)	28(68.97)	16(57.39)
AB	17(11.56)	23(15.65)	12(8.16)	52(35.37)	49(66.65)	35(57.09)
B	13(8.84)	15(10.20)	9(6.12)	37(25.16)	36(69.91)	24(59.38)
O	9(6.12)	16(10.88)	4(2.72)	29(19.73)	29(69.73)	20(59.92)
TOTAL	**52(35.37)**	**65(44.22)**	**30(20.41)**	**147(100)**	**142(96.59)**	**95(64.67)**

Key: A, AB, B and O represent the sero-grouping of the participants, CD = Complement Dependent Bactericidal Activity, CI = Complement independent bactericidal activity, A% = Average killing percentage

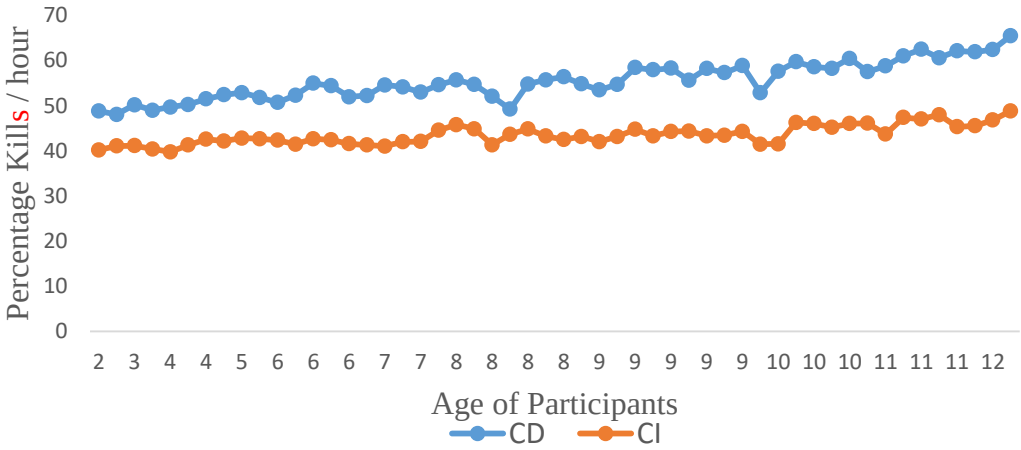

Fig. : *Staphylococcus aureus* Killing Response to Adult's Serum Bactericidal Activity with and without complement mediation

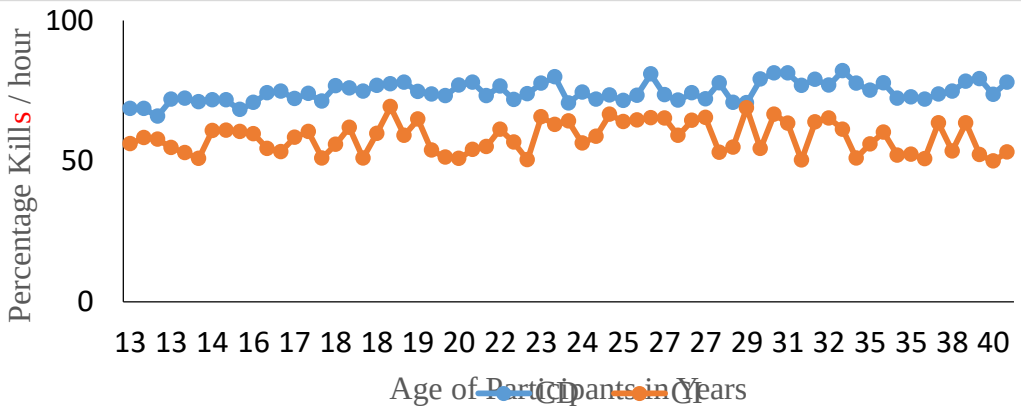

Fig. 2: *Staphylococcus aureus* Killing Response to Adult's Serum Bactericidal Activity with and without complement mediation

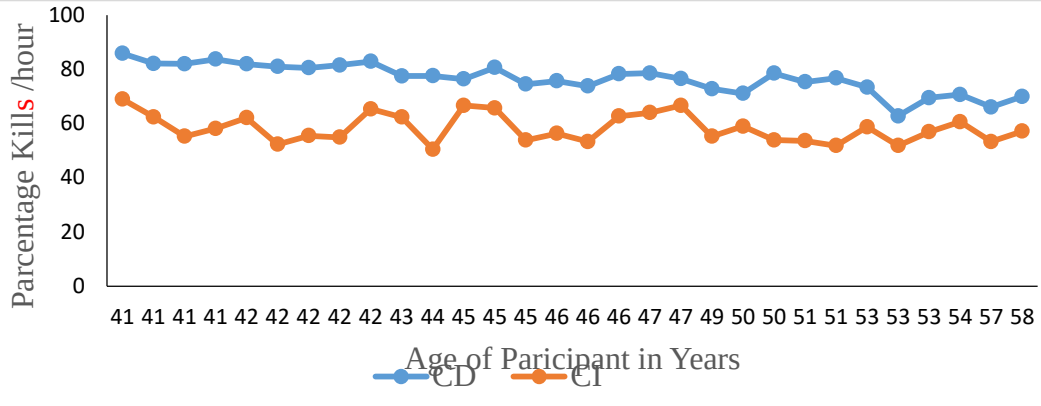

Fig. 3: *Stphylococcus aureus* killing Response to Elder's Serum Bactericidal Activity with and without complement mediation

Key: CD – Complement Dependent, CI –Complement Independent Bactericidal Activity

Discussion

The findings of this study predicted that different sera groups showed variable bactericidal activities against the studied isolates. However, participants with sero-grouping of AB revealed a less activity as compared to the other sero-groupings for both fresh and Ethylene diamine tetraacetic acid (EDTA) treated serum. Sero-groupings are frequent targets in epidemiological investigations since they are genetically determined qualities with known numerous structural manifestations among individuals and populations. Many sero-

groupings are receptors for infectious agents and their products, where they can simplify colonization or invasion or evade host clearance mechanisms. Sero-groupings can also function as false receptors, averting binding to target tissue (Teddy *et al.,* 2010).

The less bactericidal activity found in this study with regard to AB sero-grouping was in line with the finding of Olsson and Hellberg (2003), who stated that *S. aureus* is one of the chief agents associated with wounds and hospital acquired infections., it was graded the second causative agent in distressing patients, largely isolated from patients with AB sero-grouping more than other sero-groupings, these might be likely influenced by Le^a blood group antigen which serve as receptor for *Staphylococci.* It was also reported that AB sero-grouping individuals possessed more Le^a antigen when matched with other sero-groupings among populations (Najla *et al.,* 2008), although 45% of African population were AB sero-grouping these might elucidate the increased occurrence of *Staphylococcus* infection in traumatic wound patients.

Moreover, Igumbor and Osayanade (2000) reported that ABOsero-grouping is determined by its carbohydrate (Le^a antigen and others) on the surface of red blood cells which also served as a receptor for many infectious agents and facilitates their internalization into the cells.

Children fresh serum indicated a relative bactericidal activity to *S. aureus* that readily increased with increases in age that is from 2 to 12 years as matched with EDTA treated sera, although no significant difference was revealed at $p \leq 0.05$. This result is in line with the report of Mcgreal *et al.*(2012) who stated that in the absence of blood cell, the serum bactericidal activity depend on the formation of Membrane Attack Complexes (MAC), and Hogasen *et al.* (2000) who state the deficiency of C9, the major factor in MAC formation. Also, Swierzko *et al.* (2009) and Sallenbach *et al.* (2011) both reported the lack of complement recognizing molecules such as C1q, Mannose Binding Lectin (MBL), M-ficolin and L-ficolin among neonate and children.

Furthermore, both fresh and EDTA treated sera from adults and elders showed a robust capacity in the serum killing of *S. aureus*, even though no significant differences were determined in all respects. However, both fresh and EDTA treated sera from elders revealed a decline activity at 43 years of age and above. These marvel of strength and weakness were in agreement with the report of Weiskopf *et al.* (2009) and Simon *et al*. (2015) that immune system undergoes several adjustments and transformation at cellular and structural levels hence, predisposing elderly individuals to a greater risk of several infections than before. Khan *et al.* (2018) reported an incomplete serum killing percentage of *P. aeruginosa* at 120 min among people greater than 45 years. This may be the firmness of Suzanne (2001) who reported that elderly individuals are more likely not only to be hospitalized with an infectious disease, but also to die of it.

Because of this modification in the human immune system with regard to age, rate of infection and mortality in child and elderly patients are three times higher as compared to adults (Yoshikawa, 2000; Khan *et al.,* 2018). However, large number of studies have focused on imperfections in the immune system arising with aging, such as compromised phagocytosis and cytokine responses, age-related deregulations in the complement system have been largely unheeded in humans (Weiskopf *et al.,* 2009).

Likewise, existing data from other animals indirectly support the findings of this study. Hazlett *et al.* (1999) reported a weak phagocytosis activity with older mice against some

selected microorganisms. Meanwhile, a compromised function of the complement system affects several other biological functions like opsonophagocytosis and inflammation, reduced phagocytosis and other cellular responses in aged mice as described previously, indirectly supports the finding of this study. Reduced phagocytosis, nitric oxide and superoxide production with age have also been documented (De la Fuente *et al.,* 2002; Rosenberger *et al.,* 2004).

Nevertheless, the ability of EDTA treated sera to kills the potential percent of *S. aureus* exposed that the serum bactericidal activity of human is not absolutely complement-driven as reported by Oweh-Uregbe and Okoh (2008). Correspondingly, Spitzer *et al.* (2007) and Walport, (2016) reported a formidable arsenal of virulence-facilitating proteins and structures that contribute to pathogenesis of *S. aureus*, thereby avoiding immune evasion particularly the complement activation through bacterium resistant to MAC formation by targeting an initial, amplifying and pro-inflammatory steps as the peptidoglycan-rich structure of its cell wall remain developed.

Conclusion

The result of this study confirmed that human serum bactericidal activity increases with increase in age up to a certain limit (usually >45 years) where this property started to diminish. Moreover, the finding of this study supports that human serum bactericidal activity against *S. aureus* is not solely complement dependent. Moreover, ABO sero-grouping plays an important role as infection determinants, where AB sero-grouping are more vulnerable to *S. aureus* infection than other sero-groupings. This study recommend that immunotherapy strategies should be combine with antibiotic therapy, which is becoming ineffective with the passage of time due to increasing resistance of this pathogen against commonly used antibiotics.

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