

Development of Serum Bactericidal Assay for *P. aeruginosa*

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Abstract: The primary objective of this research work is to develop a serum bactericidal assay (SBA) for the quantification of bactericidal antibodies in human serum for *P.aeruginosa* (pSBA). Whole cell antigens (Heat and formalin inactivated) were generated from clinical isolates. It was screened for Anti-pseudomonas antibodies (IgG) using ELISA technique. An increased sero-reactivity with heat killed preparation 54-63% were observed than formalin killed. It may be due to the presence of different epitopes in heat& formalin inactivated antigen. ELISA positive sera were pSBA positive. No sero-cross reactivity in pSBA. No major variations in pSBAtitre with different bacterial inoculum tested. pSBA antibody driven with no interference with endogenous complement. This study demonstrated Anti-pseudomonas functional activity in human serum.

Keywords: Serum bactericidal assay, *P.aeruginosa*, Whole cell antigen, ELISA, endogenous complement, Functional antibody

1. Introduction

Pseudomonas is the most important genus in the order Pseudomonadales, the family Pseudomonaceae. *Pseudomonas aeruginosa* is a ubiquitous and remarkably versatile bacterium (Tidy, 2014). These bacteria are straight or slightly curved rods, 0.5 - 1.0 μ m by 1.5 - 5.0 μ m size and are motile by one or several polar flagella. Mucoid form (capsule) is an adaptation mechanism of *P. aeruginosa* for surviving in aquatic environments by forming a polysaccharide net/biofilm rich in alginate (Pedersen et al., 1990)

1.1 Virulence Factors

Pseudomonas has a number of virulence factors (Lyczak et al., 2000), which includes:

- Capsule, exotoxin A and various proteases that facilitated immune evasion, tissue and cellular damage for pathogenicity
- Pyocyanine - *P. aeruginosa* synthesizes a characteristic blue redox-active secondary metabolite, that is chloroform-soluble and a member of the tricyclic compounds "phenazine" called pyocyanine which is chemically, hydroxy5-methyl-phenazine (van delden 2004)

1.2 Epidemiology

P. aeruginosa flourishes in different ecological environments including soil, river, waste water, plant, animal and human. This Gram-negative bacteria lives in water and soil and acts as an opportunistic pathogen in humans and plants (Jamileh et al., 2012). Many advances in understanding the physiology and biochemistry of this organism and microbes in general have been borne off these studies. The genome of *P. aeruginosa* has an unusually large number of genes for catabolism, nutrient transport, the efflux of organic molecules, and metabolic regulation. This may explain its ability to grow in many environments and resist antibiotics (Arancibia et al., 2002).

1.3 In vitro Serology

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. 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. Although the use of such a test for assessing the bactericidal activity in a patient's serum has been widely used for 40 years, its performance, results, and interpretation have been subject to question and controversy. The bactericidal effect of normal serum on gram-negative bacteria was investigated with the objective of determining whether this antibody-complement reaction could be used for the measurement of antibody. The major variables affecting the outcome of this complement dependent reaction were explored systematically to establish the test conditions in which specific antibody was the limiting factor (Stratton 1988).

2. Review of Literature

The aim of this study is to develop a serum bactericidal assay (SBA) to evaluate the ability of the serum antibodies to kill *P. aeruginosa* by direct augmentation of complement cascade. On reviewing the literature, serum bactericidal assay model is popularly used as a platform to quantify the minimum Inhibitory concentration of antibiotics as SBT rather than SBA. Even though Gram negative bacterium owing to their cell complexity are predominantly killed by complement cascade and less through opsonophagocytosis, some strains of serum non-sensitive *Pseudomonas* strains tends to interfere with complement components. Hence, we found limited literatures pertaining to the development of SBA for *pseudomonas*.

2.1 Historical review of the SBT

Of the laboratory methods for assessing bactericidal activity, it is not surprising that the SBT has received the most attention. Tests to estimate the bactericidal activity of blood

antedate the antimicrobial era. A logical extension of this concept is the estimation of the bactericidal activity of blood during the antimicrobial treatment of serious infections.

Although (Schlichter and MacLean, 1947) are generally given credit for the SBT, they tested only the inhibitory activity of serum and did not define criteria for the determination of bactericidal endpoints. In fact, it was (Fisher, 1952) who subsequently modified Schlichter's method by sub culturing the tubes that failed to exhibit macroscopic evidence of growth after overnight incubation. Although Fisher addressed the need for determining the serum bactericidal concentration, there have been no universally accepted criteria for micro dilution or macro dilution methods for performing bactericidal testing despite the use of the SBT for 40 years. It is no wonder that critical reviews of the SBT have not found this test clinically useful and have stressed the need for standardization of the methodology.

As the SBT is presently done, there are marked differences from laboratory to laboratory in every major variable in the test. Inadequate descriptions and variation in test methods among clinical laboratories preclude objective assessment of results from clinical studies. Therefore, the National Committee for Clinical Laboratory Standards has recently published proposed guidelines for both bactericidal testing and the SBT.

These guidelines review aspects of bactericidal testing and the SBT which require standardization. The use of these guidelines through the National Committee for Clinical Laboratory Standards consensus mechanism should allow agreement about standardized methods. This will then enable the appropriate prospective clinical trials to be done to provide a rational data base for clinical interpretive criteria for the SBT in serious infections (Stratton, 1988)

Bacterial survival in the host is accompanied by the acquisition of different immune-evasive strategies. These strategies involve alteration of the bacterial surface, such as production of a capsule or immune-modulating membrane proteins, or secretion of soluble immune-evasion proteins, such as proteolytic factors. *P.aeruginosa* secretes a number of toxins and virulence factors, like elastase and alkaline protease (AprA) (Alexander et al., 2012)

2.2 Effect of serum on *Pseudomonas* species:

Pseudomonas enzymes, especially elastase and alkaline protease, were shown to have direct effect on the serum hemolytic complement as a whole, and on the two recognition molecules of complement, C1q and C3 in particular (Alexander J. laarmen, et.al, 2012). Incubation of serum with 0–50 µg/ml elastase or protease (60 min, 37°C) resulted in a dose-dependent depletion of hemolytic complement with the protease being 3–4 times more efficient than elastase. A combination of the siderophores produced by *Pseudomonas aeruginosa*, pyochelin and pyoverdine, dramatically stimulates the growth of this bacterium in medium containing human transferrin. The amount of growth stimulation observed when each siderophore was added alone was only slightly less than the

amount observed with the combination. Siderophore-defective mutants of strain PAO1 were isolated to test the effects of siderophore production on growth in transferrin and human serum (R Ankenbauer, et.al., 1985)

2.3 Effect of bacterial absorption on serum killing

The rate of intracellular bacterial lysis is largely controlled by the lysozyme concentration but complement is also necessary. Killing is closely related to complement concentration. Antibody is needed in such small amounts that it is rarely a limiting factor (Taylor, 1983)

Both lysis and killing are stopped by bentonite absorption of serum and complete return to normal is not attained by subsequent addition of egg-white lysozyme. In a lysozyme-free system lysis does occur after some delay probably due to the action of complement and antibody alone. In the presence of adequate complement and antibody the loss of complement (measured haemolytically) in bentonite treated serum is inadequate to account for the fall in bactericidal activity (Glynn and Milne, 1965). Optimal phagocytosis by PMNs required the presence of heat-stable antibody and heat labile opsonin; the activity of the latter was primarily due to the first four components of complement. Heat-stable opsonizing antibody was greatly augmented in sera of survivors of bacteremic infection. Normally functioning PMNs are essential to host defenses, since most invasive strains of *P.aeruginosa* are resistant to serum (Lowel and Young, 1972)

2.4 Inhibitors of serum killing:

Investigation has shown that the complement-dependent bactericidal activity of normal human serum towards certain organisms requires magnesium ion, and also a serum euglobulin, properdin, which is distinct from antibody and complement (Pillemer et.al., 1954; Wardlaw and Pillemer, 1956). The requirement for properdin was demonstrated by the loss of bactericidal activity following absorption of serum with the yeast cell-wall fraction 'zymosan'. The conditions of absorption were controlled so that properdin was removed without loss of component C'3. Properdin itself was eluted from the zymosan residue, and was able to restore bactericidal activity to properdin-deficient serum. The requirement for Mg ion was similarly demonstrated by the decrease in bactericidal activity following treatment of the serum with the cation exchange resin Amberlite IRC-50 (sodium cycle). Addition of MgCl₂ restored bactericidal activity to resin-treated serum. The outer membrane constitutes the main barrier to the serum killing of gram-negative bacteria. Serum-resistant cells may be sensitized to the bactericidal action of serum by treatment with Tris and EDTA, a process known to lower the permeability barrier of the outer membrane by affecting the release of approximately half of the complexed lipopolysaccharide (It appears that serum resistance does not result from a block in the activation of the complement cascade. Rather, MACs formed on the surfaces of serum-resistant strains are not effectively inserted into the bacterial membranes and are released without causing lethal damage.

2.5 Sequence of *Pseudomonas* cellular events after exposure to serum

Exposure of susceptible gram-negative cells to serum is generally followed by a period during which there is little apparent change in viability; the length of this period varies depending on the bacterial strain used, the test system, the serum concentration, and the metabolic state of the bacterial cells (D.Rowley and A.C.Wardlaw, 1958). Integration of the MAC into phospholipids bilayer areas of the outer membrane therefore disrupts outer membrane integrity and removes the barrier to hydrolysis of peptidoglycan by lysozyme. It is unlikely; however, that damage confined to the outer membrane is responsible for the lethal activity of complement (Taylor, 1983)

Complement Role

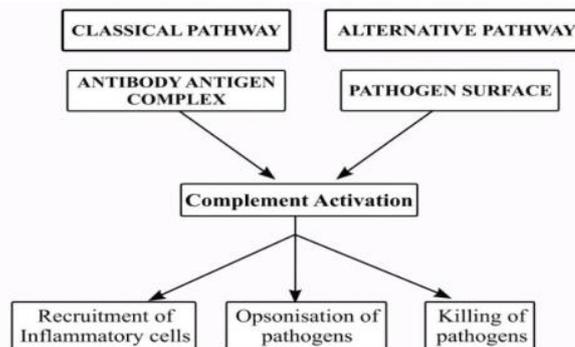
In the late 19th century, Hans Ernst August Buchner found that blood serum contained a “factor” capable of killing bacteria. In 1894, Richard Pfeiffer, a German scientist, had discovered that when cholera bacteria were injected into the peritoneum of a guinea pig immunized against the infection, the pig would rapidly die. This bacteriolysis, Bordet discovered, did not occur when the bacteria was injected into a non-immunized guinea pig, but did so when the same animal received the antiserum from an immunized animal. Moreover, the bacteriolysis did not take place when the bacteria and the antiserum were mixed in a test tube unless fresh antiserum was used.

However, when Bordet heated the antiserum to 55 degrees centigrade, it lost its power to kill bacteria. Finding that he could restore the bacteriolytic power of the antiserum if he added a little fresh serum from a non immunized animal, Bordet concluded that the bacteria killing phenomenon was due to the combined action of two distinct substances, an antibody in the antiserum, which specifically acted against a particular kind of bacterium, and a non-specific substance, sensitive to heat, found in all animal serums, which Bordet called “alexine” (later named “complement”).

The term “complement” was introduced by Paul Ehrlich in the late 1890s, according to him, the immune system consists of cells that have specific receptors on their surface to recognize antigens. Upon immunization with an antigen, more of these receptors are formed, and they are then shed from the cells to circulate in the blood. These receptors, which we now call “antibodies,” were called by Ehrlich “amboceptors” to emphasize their bi-functional binding capacity: They recognize and bind to a specific antigen, but they also recognize and bind to the heat-labile antimicrobial component of fresh serum. Ehrlich, therefore, named this heat-labile component “complement,” because it is something in the blood that “complements” the cells of the immune system.

There have been a number of studies which have examined the mechanism of serum killing of *P.aeruginosa*. Several investigators have presented evidence suggesting the importance of the classical complement pathway (CCP) activated by antibodies in the killing of some *P.aeruginosa* strains (Module Microbiology). Reports have stated that

certain strains are killed by complement activated via the alternative complement pathway (ACP) in the apparent absence of antibodies, whereas others have suggested a role for both pathways. (Schiller 1987).



Flow Chart 1: Complement pathway

Sample ID	Clinical samples	Location
1	Blood	Mylapore
2	Urine	Park town
3	Swab	Royapettah
4	Swab	Mylapore
5	Swab	Adambakkam
6	Blood	Velacherry
7	Urine	Alwarpet

Resistance of *P.aeruginosato* killing by complement and evasion from neutrophils are major virulence traits that allow this organism to survive within the bloodstream and inflamedlung. To date, the actual mechanism for complement interaction on the *P.aeruginosa* surface is not well understood.(Module Microbiology)Comparatively little work has been done on the role of anti-capsular antibodies in the serum bactericidal reaction. Antibodies to the exopolysaccharide of mucoid strains of *P. aeruginosa* are known to activate complement in the bactericidal reaction (Taylor 1983)Over the past half century an extensive literature has accumulated on the bactericidal action *in vitro* of normal serum on gram-negative bacteria (Wilson and Miles, 1955; Skarnes and Watson, 1957).

3. Methodology

3.1 Collection of sample:

Samples collected from clinical laboratory, Hospitals totally 7 samples and transported in Stuart medium.

3.2. Isolation of pathogen:

The sample was serially diluted and plated onto nutrient agar plates, kept for incubation at 37°C for 24 hours.

3.3 Characterization of the *Pseudomonas* clinical isolate

A loopful of the inoculum from incubated nutrient agar plates was streaked on to selective media *Pseudomonas* isolation agar.Preliminary morphological and biochemical tests such as Gram staining, motility, Indole, Methyl red, Vogesproskauer, Citrate, Triple sugar iron, Urease, Nitrate reduction, Catalase and Oxidase were performed using

standard methodology (Cappuccino, 1999) and results were tabulated.

3.4 Collection of serum sample for serological tests

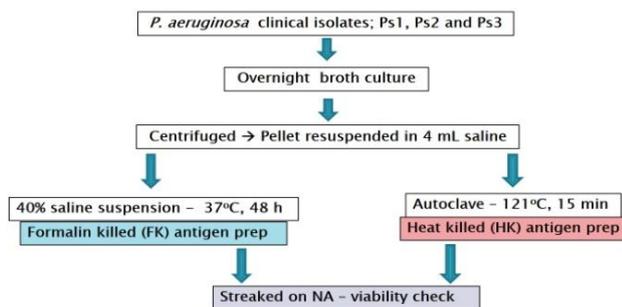
The serum samples were collected from various hospitals and diagnostic laboratories in and around Chennai. Serum samples were collected from laboratory through lab technicians and stored in eppendorff and kept in ice. Totally 22 multiple aliquots of serum were prepared.

3.5 Elisa for anti *Pseudomonas* antibodies in human serum

3.5.1 *P. aeruginosa* antigen preparation for ELISA

Human serum was screened for the presence of anti-pseudomonas antibodies using ELISA antibody capture technique. To capture the pseudomonas specific serum antibodies, two different whole cell antigenic preparations were made from three *P. aeruginosa* clinical isolates, Ps1, Ps2, Ps3. Protocol adopted for the generation of heat killed and formalin killed antigen to be used for antibody capture in ELISA is given in Flow Chart 2.

Whole cell antigens preparation for ELISA

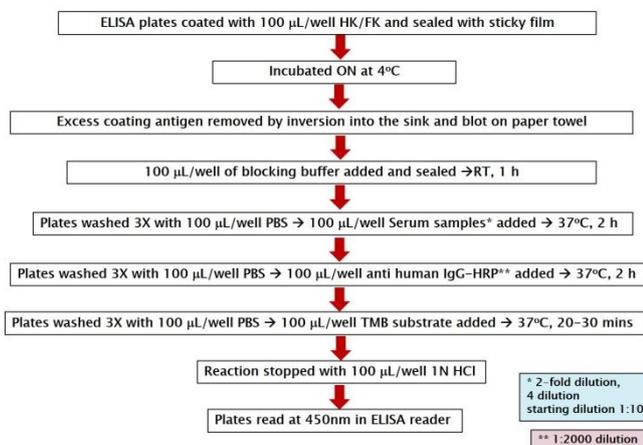


Flow Chart 2: *P. aeruginosa* antigen preparation for ELISA

3.5.2 *P. aeruginosa* ELISA

To screen a panel of human serum (n=20) for pseudomonas specific antibody (IgG), ELISA antibody capture technique was used with heat killed and formalin killed whole cell antigen preparations of 3 pseudomonas clinical isolate viz., Ps1, Ps2, Ps3. ELISA protocol is given in Flow Chart 3.

ELISA



Flow Chart 3: *P. aeruginosa* ELISA

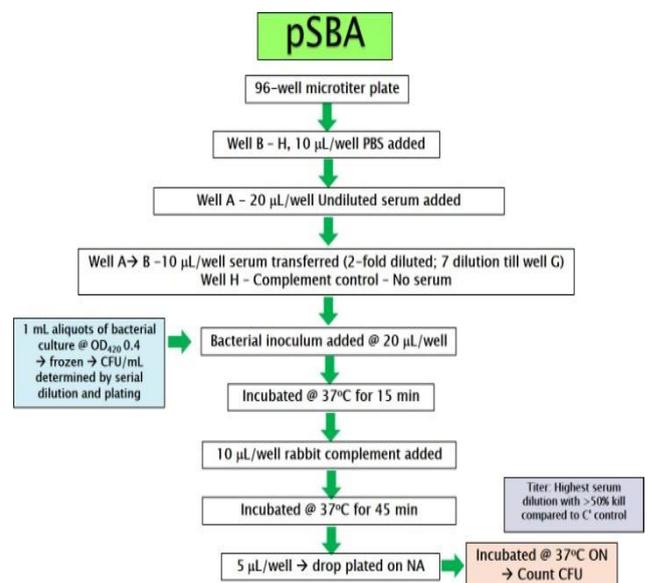
As there is no standard reference serum available to generate concentration vs OD₄₅₀ calibration curve, OD₄₅₀ for each serum sample was used for comparative analysis. In order to normalize the impact of dilution on the overall antibody concentration in various serum samples, OD₄₅₀ was converted and expressed as weighted average (WA) using the following formula:

$$WA = \frac{\sum(a_1 \times d_1, a_2 \times d_2 \dots a_n \times d_n)}{\sum(d_1, d_2 \dots d_n)}$$

WA = Weighted average; a₁=OD₄₅₀ for first dilution; d₁ = dilution factor; Σ = Sum

3.6 *Pseudomonas* serum bactericidal assay

Serum bactericidal assay for pseudomonas was developed as described in the flow chart 4. Based on the ELISA observations, only two *P. aeruginosa* strains, Ps1 and Ps3 were tested for serum bactericidal activity against two positive and two negative human sera.



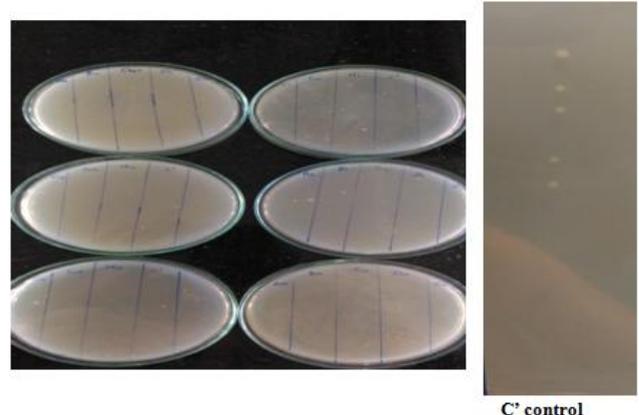
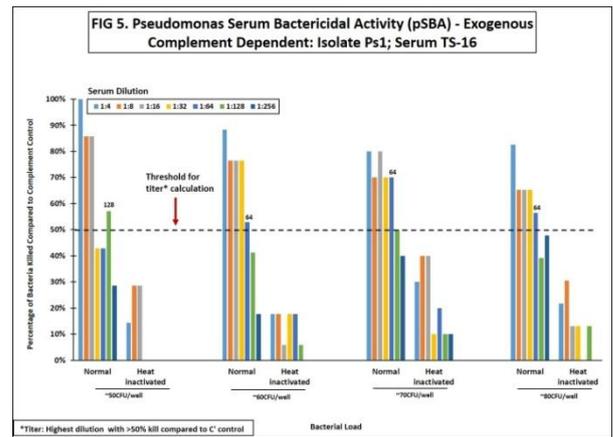
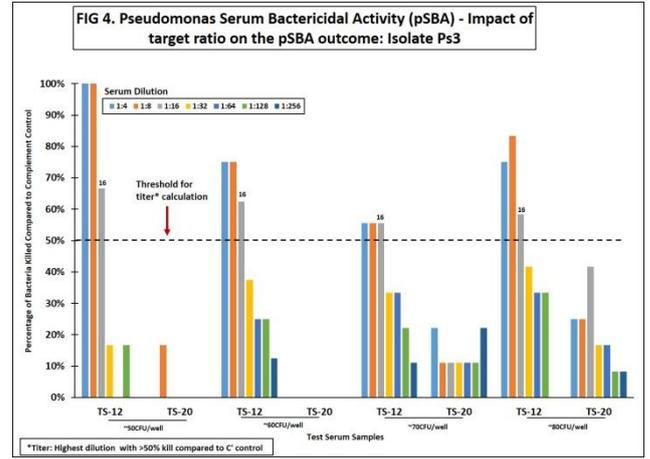
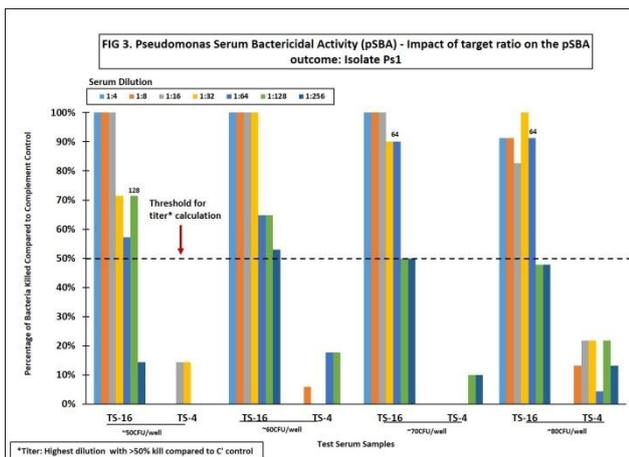
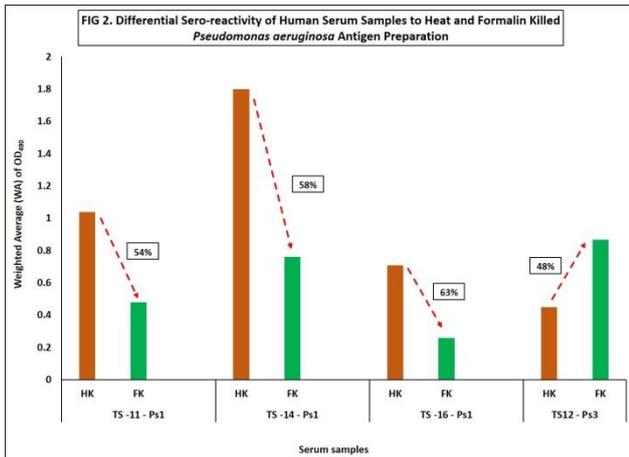
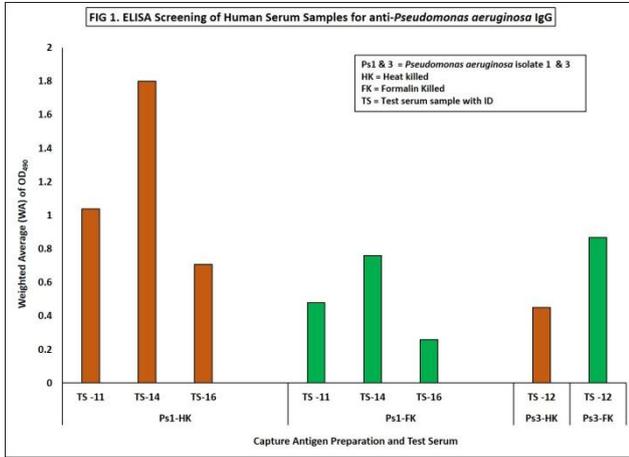
Flow Chart 4: *P. aeruginosa* SBA

Two important assay variables were included for optimization. The first variable is the target antibody ratio. While serum samples were diluted 2-fold from undiluted samples through 7 dilutions, the bacterial target was inoculated in 4 different counts, 50, 60, 70 and 80 CFU/well. The second important variable is the endogenous complement source that may contribute to the background killing of the target bacterium. This question was addressed by including a heat inactivated (56°C for 30 min in a water bath) positive serum control. Inclusion of complement control served two purposes. While the complement control along with negative control gave as data to set the baseline (50% killing), it also enabled us monitor the target bacterium for direct complement inactivation in which case we would observe a flat line in killing with no serum dilution dependent variation in killing in comparison to C' control.

4. Result

Table 1

Isolates	Sample ID
Ps1	6
Ps2	3
Ps3	2



5. Discussion

The colonies from the nutrient agar plates were confirmed as *Pseudomonas aeruginosa* by selective media plating (Plate 1 - 3) and biochemical tests (Table 1).

Three *Pseudomonas aeruginosa* clinical isolates (Ps1, Ps2 and Ps3) were selected for antigen preparation. Heat and formalin killed antigen preparations were generated from these *Pseudomonas* isolates as described in the methodology. These whole cell antigen preparations were coated in the ELISA plate as capture antigens. Human sera (n=20) obtained from a clinical laboratory were screened for anti-pseudomonas antibodies (IgG) using these ELISA plates. Among the 20 test serum samples, three (TS-11, TS-14 and TS-16) were found to be reactive with both heat and formalin killed preparations of Ps1 (FIG 1). Human serum sample TS-12 was reactive with the antigenic preparations

(both heat and formalin killed) of Ps3. None of the test serum samples reacted with Ps2 antigenic preparations. For Ps1, serum sample TS-14 exhibited maximum sera-reactivity in both heat and formalin killed preparations (Weighted Average (WA) of $OD_{490} = 1.8$ and 0.8 respectively) followed by TS-11 (WA = 1.0 and 0.5 respectively).

Data presented in FIG 2, reveals a clear difference in sera-reactivity of test serum between heat and formalin killed preparation. For Ps1, all three positive serum samples demonstrated an increase in sera-reactivity with heat killed preparations compared to formalin killed (54 – 63%). On the other hand, TS-12 the only serum sample with sole reactivity with Ps3 whole cell antigenic preparations, exhibited a 48% reduction in reactivity with heat killed preparation compared to formalin killed preparations. Is this difference in reactivity, 1) contributed by a simple changes in the epitope densities due to different treatments of the bacterial cell (heat and formalin); 2) Or does different inactivation procedures resulting in the exposure of different epitope that were captured by the polyclonal human serum; 3) Or is this due to changes in the conformation of the epitopes in response to heat or formalin that impacts the affinity kinetics (Km) of the antibodies in the human serum? Some of these questions will be addressed in future with additional experiments. This includes, homologous and heterologous competition experiments, and antibody avidity analysis

The primary objective of this research work is to develop a serum bactericidal assay (SBA) for the quantification of bactericidal antibodies in human serum for *P. aeruginosa* (pSBA). One of the critical variables in pSBA is the target (bacterial) ratio in the reaction. Hence, the bacterial inoculum was optimized according to the method described in the previous section with a positive and negative serum and the results presented in FIG 3 – 4). With the pSBA titer threshold set at 50% bacterial kill compared to complement control (without serum), the positive control reported a pSBA titer of 64-128 at 4 different inoculum load (~50, 60, 70, and 80 CFU/well) of Ps1. Similarly for Ps3, the positive control has returned a pSBA titer of 16 in all 4 inoculum load (FIG 4).

Human serum contains endogenous complement that may contribute some background killing of the bacterium included in the assay mixture. This may not adversely affect the outcome of the assay as the serum gets diluted resulting in the endogenous complement also even if present. Nevertheless, the presence of endogenous may alter the assay background making it difficult to standardize. We tested the human serum (positive control) tested in pSBA for active endogenous complement by including a heat inactivated pair alongside the normal serum. As shown in FIG 5 and 6, the test sera have not exhibited any endogenous complement activity. This is evident with the disappearance of titer in the heat treated serum without exogenous complement compared to normal serum with exogenous complement. We anticipate the reappearance of bactericidal activity in the heat inactivated serum if supplemented with exogenous complement. This will be carried out in future studies.

6. Summary and Conclusion

The clinical sample obtained from various hospitals & laboratories were processed, *P. aeruginosa* was isolated from nutrient agar plate on selective medium. Pseudomonas isolation agar. Heat killed and formalin killed lysate were prepared by an appropriate technique as mentioned in methodology. Serum samples (n=22) collected from various laboratories were screened for pseudomonas specific IgG using direct antibody capture ELISA protocol. Serum samples TS11, TS14, and TS16 had IgG for Ps1 and serum sample TS12 for Ps3. No ELISA sero-reactivity was recorded for Ps2 in any of the test serum samples. A clear distinction among the two different antigen preparations was recorded within the Ps isolates. While serums for Ps1 were more reactive with HK prep, Ps3 was highly reactive to FK suggesting differential exposure of antigens in response to inactivation procedures. Additional experiments as suggested in the results and discussion may provide clarity in this regard.

SBA for the Ps1 and Ps3 looks promising in terms of antibody and complement dependent nature without interference from the endogenous complement or complement lytic activity of the target bacterium. Further optimizations will enable pSBA characterized as a robust serological assay for pseudomonas.

To conclude, this study has demonstrated the feasibility of complement driven *in vitro* SBA for *P. aeruginosa*. *In vitro* serological correlate of protection such as pSBA are important for the development and evaluation of pseudomonas vaccines.

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