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ORIGINAL ARTICLE

Characterization of complement activation on the surface of *klebsiella pneumoniae*

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ABSTRACT

Background: *Life threatening infections caused by the opportunistic pathogen K. pneumoniae are one of the most serious infections that elevate mortality rate in hospitals, worldwide. K. pneumoniae develops several mechanisms for avoiding killing by the complement system for survival in the host. The complement system is the first line of immune defense against different bacteria including K. pneumoniae*. ***Objective:*** *In this study we assessed the role of complement system in killing of both serum resistant and serum sensitive strains of K. pneumoniae.* ***Methodology:*** *Six clinical isolates of* K. pneumoniae *were collected from urine, sputum and the blood samples from patients in Mansoura Hospitals University. Serum bactericidal effect was assessed and confirmed by Turbidimetric method, Complement mediated lysis, complements C3b deposition and fH binding were assessed by ELISA.* ***Results:*** *our results demonstrated that complement mediated killing of K. pneumoniae is driven via the Alternative pathways, it was found that a lower C3b deposition via AP and a higher fH binding to surface of serum resistant K. pneumoniae strains compared with serum sensitive K. pneumoniae* ***Conclusions:*** *This study explains the role of complement system in killing K. pneumoniae and how serum resistant strains escape the immune defense via sequestering complement factor H.*

INTRODUCTION

Klebseilla Pneumoniae is one of the Gram negative opportunistic pathogen causing hospital-acquired infections in immunocompromised patients including bloodstream infections, respiratory tract infections, as well as urinary tract infections [1]. Endophthalmitis, pneumonia, necrotizing fasciitis, non-hepatic abscess and meningitis are examples of community acquired infections caused by *K. pneumoniae* [2, 3].

K. pneumoniae as many pathogens have developed several evasion strategies for survival in different hosts [4]. In consequence of wide spread infections caused by multi-drug resistant strains, it becomes important to know how *K. pneumoniae* can invade tissues and escape from host immune responses. Complement system plays a main role in recognition, opsonization, and killing of different pathogens including *Klebsiella* species [5, 6]. Complement system is activated when complement recognition molecules attach to the bacteria surface, this leads to opsonization of the pathogen with subsequent killing via phagocytosos or cell wall disruption via formation of membrane attack complex[7].

There are three pathways that activate the complement system; the classical (CP), the lectin (LP) and the alternative pathway (AP). The CP is activated when the recognition subcomponent C1q attaches to immune complexes with subsequent activation of C1r and C1s. Activated C1s cleaves C4 into C4a and C4b followed by cleavage of C4b-bound C2 to generate the C3 convertase (C4b2a). C3 convertase cleaves C3 into C3a and C3b, the essential opsonins of complement system. The LP is initiated through the recognition of pathogen-associated carbohydrate or acetylation patterns present on microbial surfaces, by the carbohydrate recognition molecules including; mannan-binding lectin (MBL), collectins (CL-10, CL-11 and CL-12), and ficolins (L-ficolin, M-ficolin, and H-ficolin). The carbohydrate recognition molecules of the LP form complex with three serine proteases, these proteases include; MASP-1, MASP-2, and MASP-3. Only MASP-2 cleaves C4 and C4b-bound C2 and generates C4b2a, the CP and the LP C3 convertase [8-10]. The AP is the third pathway which is activated in consequence of spontaneous activation of C3 that generates C3 (H2O) which attracts Factor B that results in formation of C3 (H2O)B zymogen complex. Factor B is cleaved via factor D to form C3 convertase C3bBb which can convert more C3 into C3a and C3b. When complement system is activated, proteins of complement C5b, C6, C7, C8, and replicates of C9 (C5b-9) aggregate on the surface of bacteria and form membrane attack complex (MAC) that kills bacteria by making pores in outer membrane specifically in Gram-negative bacteria. The release of C3a and C5a anaphylatoxins enhance phagocytes and initiating local

inflammation [11-16]. Complement system is regulated via cell-bound regulators (CR1, MCP, CRIg, and DAF CD59) and plasma proteins (C1 inhibitor, factor I, C4bp and factor H) to down regulate the activation of complement cascade [17-21]. Factor H is the main fluid phase regulator of the alternative pathway. It accelerates the decline of the alternative pathway C3 convertase (C3bBb) via binding to C3b and removing it from the complex (C3bBb). Factor H acts as a cofactor in the factor I mediated conversion of C3b to iC3b, C3c and C3dg [22].

The tendency of bacteria to sequester complement regulators to their surface, resulting in complement system inactivation, is the most common mechanism of resistance of bacteria killing by complement system [12, 15].

In our research we assessed different pathways of complement activation on the surface of *K. pneumoniae* and how the bacteria resist complement mediated killing.

METHODOLOGY

Ethical Statement:

In our research, normal human sera were collected from healthy adult volunteers after given written consent. The research was approved by the Ethics Committee in Faculty of Pharmacy, Mansoura University, Egypt (Code Number 2021-230).

Buffers:

The buffers used in our research were :barbital buffered saline (BBS) (145 mM NaCl and 4 mM barbital, pH 7.4), EGTA (10 mM ethylene glycol tetraacetic acid with BBS), Tris buffered saline (TBS) (140 mM NaCl, 10 mM Tris-HCl pH 7.4) and coating buffer (35 mM NaHCO315 mM Na2CO3, pH 9.6).

Bacterial strains

Six clinical isolates of *K. pneumoniae* were collected from urine, sputum and the blood samples from patients in Mansoura University Hospital, Egypt. Bacteria were isolated and identified morphologically and biochemically.

Serum bactericidal assay (SBA)

K. pneumoniae isolates were grown on blood agar at 37°C for overnight. Bacterial cultures were collected, washed twice using BBS then adjusted to a final concentrations of 1×10^7 CFU mL^{-1}. 1×10^6 CFU was incubated with different concentration of normal human serum (NHS) in BBS at 37°C with gentle shaking [23]. After 2 hours, samples were taken and plated out on nutrient agar plate for overnight at 37°C. Serum bactericidal activity was calculated by measuring the decline in the viable bacterial count recovered after 2 hours incubation with NHS compared to the original bacterial count at zero time point.

Turbidimetric assay:

Serum sensitivity of the *K. pneumoniae* was confirmed using turbidimetric assay[24]. Bacteria were grown first on blood agar plate then sub-cultured into nutrient broth overnight at 37°C. 1×10^6 CFU were incubated with 40 % NHS diluted in BBS in 96 well plates (round bottom) with gentle shaking at 37° C for 3 hours. Absorbance at 630 was recorded at different time points.

Complement mediated lysis assay

$1x10^6$ CFU were incubated with 40% NHS in BBS buffer (all complement pathways are active), 40 % NHS in EGTA buffer (to support alternative pathway activity only), 5% NHS with BBS buffer (only lectin and classical pathway are active), and with heated in-activated serum as a control. After 2 hours incubation at 37° C with gentle shaking, samples were collected, serially diluted and then plated on nutrient agar plates. After overnight incubation at 37° C viable bacterial count was calculated [25 26].

Complement C3b deposition and fH binding assay

An ELISA plate was coated with 100 µL of 10 µg/mL zymosan or formalin-fixed *K. pneumoniae* (OD600=0.5) in coating buffer. The residual binding sites were blocked with 250 µL of Tris buffered saline (TBS) (140 mM NaCl, 10 mM Tris-HCl, pH 7.4) containing 1% (w/v) bovine serum albumin (BSA) at room temperature for 2 hours, then, washed by wash buffer (TBS plus 0.05% Tween 20 and 5 mM CaCl2). NHS was diluted in BBS (145 mM NaCl, 4 mM barbital, 2 mM CaCl2, 1 mM MgCl2, pH 7.4), added to the plates and incubated for one hour at 37°C. The plates were washed again, and bound C3b or fH were detected using either rabbit anti C3c or rabbit anti fH antibodies (Dako, Denmark). After one hour incubation at room temperature, an alkaline phosphatase conjugated goat anti rabbit IgG (Sigma, USA) was added and incubated for another one hour at room temperature then washed by wash buffer. Bound antibody was detected using the chromogenic substrate p-Nitrophenyl phosphate pNPP (Sigma). The absorbance was measured at 405 nm using Biorad ELISA micro-titre plate reader. In a parallel experiment, NHS was diluted in EGTA buffer and added to the plate then incubated for one hour at 37°C and the AP way mediated C3b deposition was measured as mentioned above.

RESULTS

Normal human serum sensitivity of *Klebseilla pneumoniae* isolates from patients.

To assess the activity of NHS to kill *K. pneumoniae*, different concentrations of NHS were used in a serum bactericidal assay (SBA). The rate of serum

bactericidal activity increased towards some *K. pneumoniae* strains as the serum concentration increased (Fig. 1A). Three different serum resistant isolates (R1, R2 and R3) and 3 serum sensitive isolates (S1, S2 and S3) were chosen using the previous method to complete this study. To confirm the identity of serum resistant and serum sensitive isolates, serum sensitivity of the clinical *K. pneumoniae* isolates was measured by incubation of the bacteria with 40% NHS for 2 hours with gentle shaking and OD630 was measured every 30 minutes. Again, serum resistant strains survived the high serum concentration and the OD630 of their cultures started to increase by time. Serum sensitive strains did not multiply and killed by the high serum concentration where the OD630 of the bacterial culture did not show any increase by time (Fig. 1B).

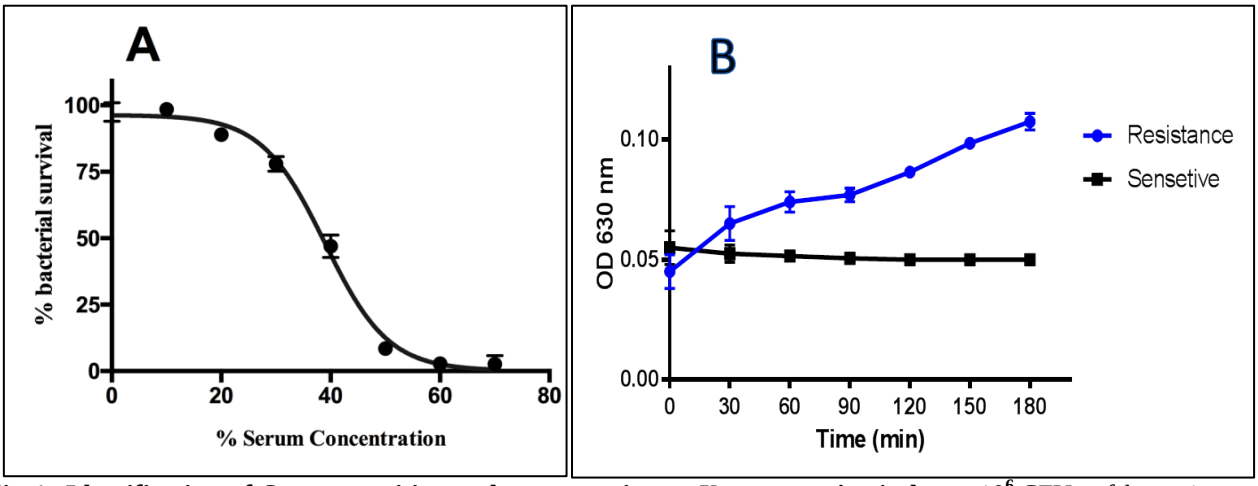

Fig 1: Identification of Serum sensitive and serum resistant *K. pneumoniae* isolates. 10^6 CFUs of bacteria were incubated with different NHS concentrations at 37°C for 2 h with gentle shaking. Samples were taken and viable bacterial count was calculated (A). Serum sensitivity was confirmed using turbidimetric assay, where bacteria was incubated with 40% NHS and bacterial growth was estimated by measuring the OD630 at different time points (B). Results are means ± SD of duplicates and are representative of three independent experiments.

Alternative pathway has a main role in opsonization and killing of *K. pneumoniae.*

Low concentration of serum (5% NHS) in BBS supports both the lectin and the classical pathways while the alternative pathway is driven by high serum concentration in presence of Mg^{+2} supported by BBS buffer with 10mM EGTA. Both the lectin and the classical pathways show a significant high level of complement C3b deposition on the surface of *K. pneumoniae* but they have no bactericidal effect on the pathogen (Fig.2A). On the other hand, a robust complement C3b deposition via the alternative pathway was observed on the surface of the bacteria which is related to a significant bactericidal activity against *K. pneumonaie* (Fig.2B). These results clearly show that the alternative pathway has the main role in opsonization of *K. pneumoniae*. To assess the role of the AP in killing *K. pneumoniae*, bacteria was incubated with either 40% NHS in BBS with Ca+2 and Mg+2 or with 40% NHS in BBS with EGTA and Mg+2 to target the AP only. Interestingly the results showed no significant difference in bacterial killing when using both conditions. Using EGTA buffer with Mg+2 solely allow the AP activity indicating the essential role of the AP in killing of serum sensitive *K. pneumoniae* compared to CP and LP. On the other hands, serum resistant strains were not killed when using high serum concentrations implying that these strains developed a mechanism to resist complement mediated bacterial killing. Our results showed that serum resistant strains showed a significant higher binding with complement fH compared to serum sensitive strains.

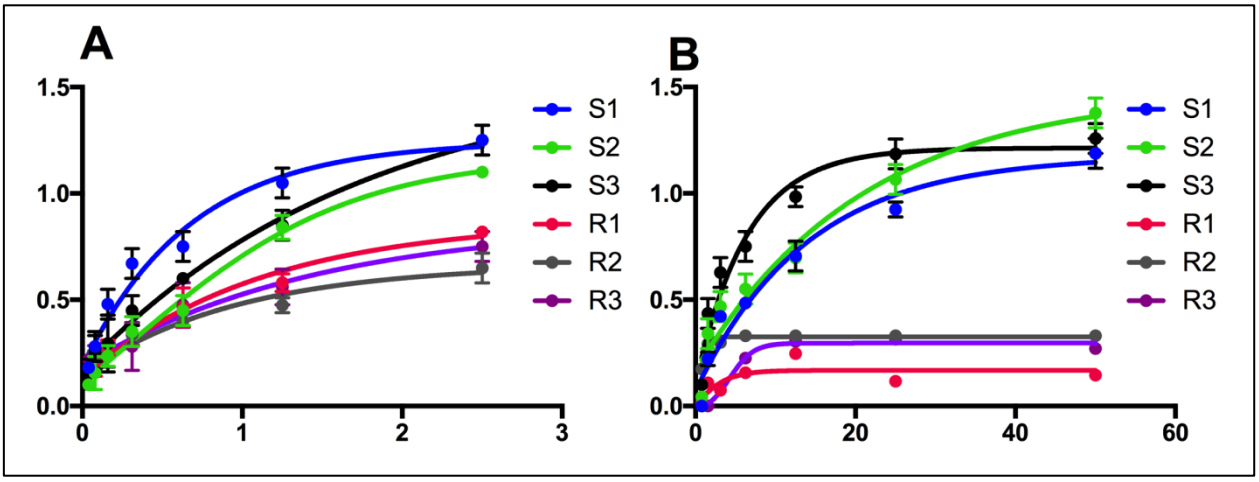

Fig.2: Complement C3b deposition on *K. pneumoniae*. A microtitre ELISA plate was coated with formalin fixed *K. pneumoniae*. Complement C3b deposition under LP and CP or AP conditions were assayed. A significant high level of complement C3b deposition via the LP/CP was observed on both serum resistant and serum sensitive isolates (A). A significant C3b deposition via the AP on serum sensitive isolates was observed while no C3b deposition was detected on the surface of serum resistant isolates (B). Results are means ± SD of duplicates and are representative of three independent experiments.

A significant high level of bacterial killing was observed when using 40% NHS in BBS but not with 5% NHS. Interestingly, the same level of robust killing was observed under AP conditions when using 40% NHS in BBS-EGTA buffer. The experiment was performed using 3 different *K. pneumoniae* isolates (A-C) (Fig 3).

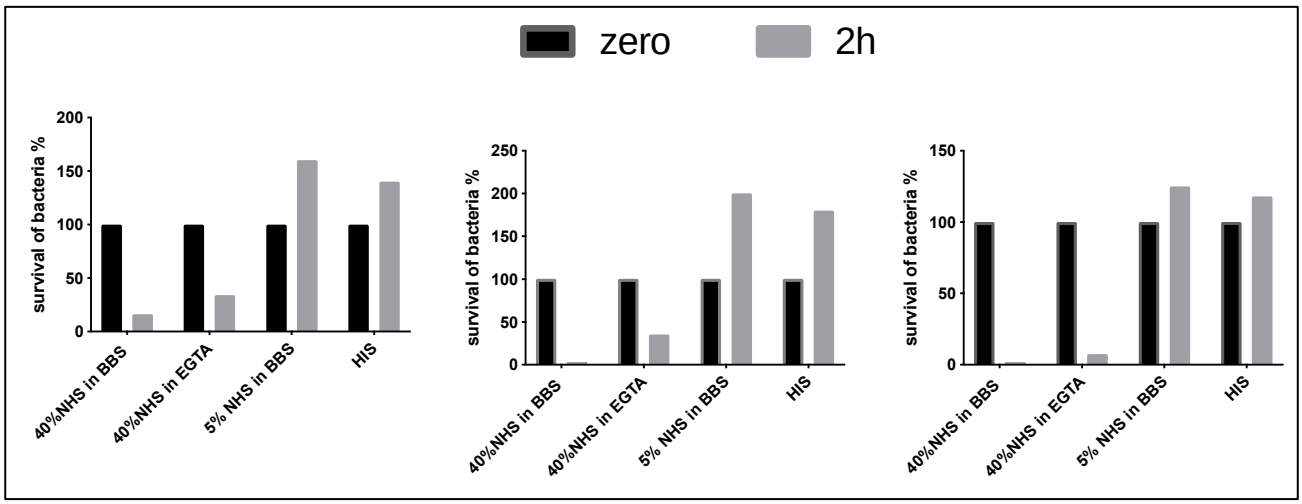

Fig. 3: Effect of complement mediated lysis on killing of *Klebseilla pneumoniae*. 10^6 cells of *K. pneumoniae* were incubated with 40% and 5% NHS in BBS at 37°C for 2 h. In a parallel experiment *K. pneumoniae* was incubated with 40 %NHS in BBS with Mg^{+2}-EGTA. Bacteria incubated with 40% heat inactivated serum were used as a control. Samples were taken and viable bacterial count was calculated.

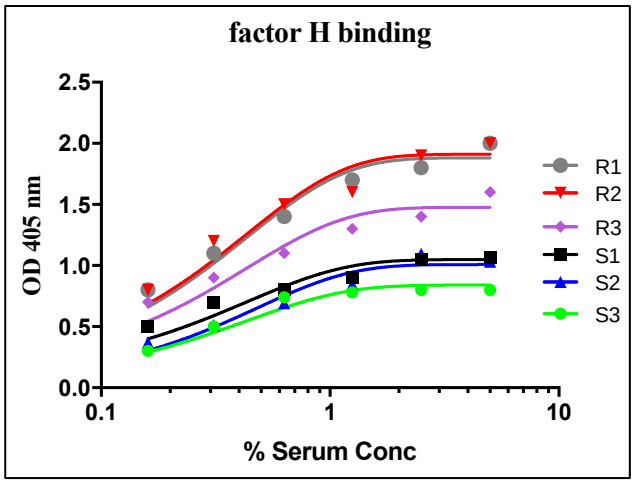

Fig. 4: Serum sensitive strains (S1, S2 &S3) of *K. pneumoniae* showed a significant higher binding to complement factor H compared to serum resistant strains (R1, R2 &R3).

DISCUSSION

The first line of innate immunity is the complement system that its main function includes bacterial detection, opsonization, and killing. *K. pneumoniae* has developed many escaping mechanisms from complement mediated killing to survive host immune defenses[4]. In this study we showed that a strong complement C3b deposition via all pathways with serum sensitive strains. Interestingly, a significant low complement deposition was observed in case of serum resistant strains via the AP indicating that serum resistance strains developed a mechanism to overcome complement activation via the AP. Although all pathways participate in opsonization as well as activation of complement on the surface of the bacteria, our results demonstrated that complement mediated killing is driven via the AP not the CP or the LP. These results were in agreement with a previous report about the role of the AP in killing *K. pneumoniae* infection [27]. Alternative pathway is regulated via plasma protein complement factor H that cleaves deposited C3b and inhibits formation of AP C3 convertase (C3bBb) on the surface of *Klebsiella*. [28]. many types of bacteria have developed the same mechanism via attraction of factor H to their surface and escape from complement mediated killing like *Neisseria species* [29], *Streptococcus pyogenes*[30], *Streptococcus pneumoniae*[31], *Borrelia species*[32], *Acinetobacter baumannii* [33] and *Enterococcus faecalis*[34].

In our study, a lower C3b deposition via AP and a higher fH binding to surface of serum resistant *K. pneumoniae* strains compared to serum sensitive *K. pneumoniae* was observed. This finding confirms our suggestion that *K. pneumoniae* escape from AP activation by sequestering fH from plasma of the host to its surface.[35] A previous study reported that individuals with low serum level of complement fH in blood are more susceptible to infection caused by various types of pathogens where fH accelerate C3b decomposition on the surface of *such types of microorganisms* [36]. Complement C3b deposition via CP and LP was appeared on the surface of *K. pneumoniae*, this proves that the important of synergistic effect of 3 pathways together to enhance complement C3b deposition with subsequent killing of bacteria via the production of MAC. To further characterize which bacteria component is responsible for binding with the host fH, more studies will be done in that area.

CONCLUSION

This study explains the role of complement system in killing *K. pneumoniae* and how serum resistant strains escape the immune defense via sequestering complement factor H.

Assignment Conflict: No significant competing financial, professional or personal interests that might have influenced the performance or presentation of the work described in the manuscript

- The authors declare that they have no financial or non-financial conflicts of interest related to the work done in the manuscript.
- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher

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