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Age-related variations in the *in vitro* bactericidal activity of human sera against *Pseudomonas aeruginosa*

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Abstract

The human serum is a vital component of the innate immunity of the host that acts as the first line of defence against invading pathogens. A key player in serum-mediated innate immune defence is a system of more than 35 proteins, collectively named as the complement system. After exposure of the pathogen, these proteins are activated in a cascade manner, ultimately forming a membrane attack complex (MAC) on the surface of the pathogen that directly lyses the bacterial cell. Formation of the MAC can be demonstrated in vitro by using serum bactericidal assay (SBA) that works in the absence of cellular components of blood after incubating the serum along with bacteria. Here, we describe the age-related differences in the bactericidal activity of human serum against Pseudomonas aeruginosa, an opportunistic human pathogen causing an array of hospital and community-acquired infections.

We demonstrate that adult sera were highly effective in the in vitro killing of Pseudomonas aeruginosa as compared to children and the elderly ($p < 0.0001$). Sera from children were seriously compromised in the killing P. aeruginosa, whereas elderly sera showed a reduced level of killing. Data revealed a positive correlation between age and serum-killing with higher coefficient of determination values of 0.34, 0.27, and 0.58 and p values of < 0.0001, < 0.001, and < 0.0001, respectively, after 60, 90, and 120 minutes of incubation. Hence, our study highlights the age-related difference in the bactericidal activity of human sera. We conclude that sera of children are totally compromised, whereas elderly sera are only partially compromised, in the killing of P. aeruginosa.

Key words: *complement system, Pseudomonas, serum bactericidal assay.*

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Introduction

Pseudomonas aeruginosa is a Gram-negative bacterium causing an array of infections in animals and humans. In humans, it is considered as a common opportunistic pathogen causing a variety of diseases in general population. It is a part of normal human microflora; however, it becomes more harmful when an individual becomes immunocompromised [1]. It is believed to be one of the most frequent pathogens responsible for nosocomial infection in the blood, lungs, urinary tract and wounds. In addition, it may cause community-acquired infections outside hospitals including pneumonia, skin rashes, external ear canal infections, eye and heart valve infections [2]. For the effective treatment of *P. aeruginosa* infection, an appropriate empirical antimicrobial therapy is important [3, 4]. However, *P. aeruginosa* poses a great healthcare challenge due to its antibiotic resistance, which is increasing with the passage of time, rendering the commonly used antibiotics ineffective against this bacterium [5–7].

The innate immune system plays a vital role in defense against *P. aeruginosa* invasions [8]. Among different effectors of the innate immunity, the complement system plays a critical role in the clearance of *P. aeruginosa* infection [9–14]. Complement, a collective term used for more than thirty serum proteins, is an integral part of the innate immune system. In addition to its role in innate immunity, it also acts as a bridge in regulating different functions of adaptive immunity. The major biological functions of the complement system include enhanced inflammation and phagocytosis along with direct cell lysis by the formation of MAC on the surface of pathogens [15].

The bactericidal effect of serum is an essential innate immune mechanism of the host that provides protection

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against harmful bacteria. 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). Several studies have investigated the bactericidal activity of serum against different pathogens [16–20]. It is widely known that the *in vitro* killing of bacteria by serum is mainly complement-driven [16, 21, 22].

A number of recent studies have established the fact that complement-mediated bactericidal activity helps in the protection of the human body against disease-causing organisms, including *P. aeruginosa* [8, 12, 13]. *In vitro* studies, using healthy human sera, reflected the significance of both alternative and classical pathways in the complement-mediated killing of serum-sensitive strains of *P. aeruginosa* [23–26]. On the other hand, sera of complement-deficient patients demonstrated weak killing activity of *P. aeruginosa* [27–29], which further supports the fact that serum bactericidal activity is mostly complement-driven. Several murine model studies of *P. aeruginosa* infection have described the important contributions of the classical and alternative pathways in complement-mediated phagocytosis of *P. aeruginosa* in lung infections [13, 30, 31]. Similarly, the importance of the lectin pathway has been reported in burn wounds infection of *P. aeruginosa* a rodent model, describing the promising role of mannose-binding lectin (MBL) in recognizing and eradicating this bacterium [32]. In addition, MBL deficiency has also been linked with the earlier infection of *P. aeruginosa* and increased rate of death in patients with cystic fibrosis [33]. Although, previous *in vitro* and *in vivo* studies have clearly established an association of complement deficiencies with the killing of *P. aeruginosa*, the association of age with the serum killing of *P. aeruginosa* has not been described. Age-dependent variations in serum killing of *P. aeruginosa* have thus been investigated in the present study to describe the association between age and serum bactericidal activity against *P. aeruginosa*.

Material and methods

Subjects

Subjects of the current study were healthy individuals with no prior history of *P. aeruginosa* infection. Subjects were categorized into three groups, children, adults and

Table 1. Detailed description of age and gender of subjects included in this study

Children			Adults			Elderly		
Volunteer No.	**Age**	**Gender**	**Volunteer No.**	**Age**	**Gender**	**Volunteer No.**	**Age**	**Gender**
1	50 days	Female	24	11 years	Female	43	41 years	Female
2	6 months	Male	25	15 years	Female	44	41 years	Male
3	12 months	Male	26	18 years	Female	45	42 years	Male
4	14 months	Female	27	18 years	Male	46	42 years	Male
5	16 months	Male	28	19 years	Female	47	43 years	Female
6	18 months	Male	29	20 years	Male	48	44 years	Male
7	18 months	Male	30	20 years	Male	49	45 years	Male
8	2 years	Female	31	20 years	Male	50	45 years	Male
9	2 years	Female	32	23 years	Female	51	45 years	Male
10	3 years	Male	33	23 years	Male	52	45 years	Male
11	4 years	Female	34	23 years	Male	53	48 years	Male
12	5 years	Male	35	24 years	Male	54	49 years	Female
13	5 years	Male	36	25 years	Male	55	56 years	Female
14	6 years	Female	37	26 years	Female	56	57 years	Male
15	6 years	Male	38	27 years	Female	57	59 years	Male
16	7 years	Female	39	28 years	Male	58	60 years	Female
17	8 years	Female	40	30 years	Male	59	65 years	Female
18	8 years	Male	41	35 years	Male	60	67 years	Female
19	9 years	Female	42	40 years	Male	61	80 years	Male
20	9 years	Male				62	85 years	Female
21	9 years	Male						
22	10 years	Male						
23	10 years	Male						

elderly. Twenty subjects from each group were investigated for serum bactericidal activity of *P. aeruginosa*. Subjects between 0–10 were placed in children, 11–40 were placed in adults and 41 and above were placed in elderly. A detailed description of age groups in presented in Table 1. All the procedures were performed in the Microbiology Laboratory of the Pakistan Institute of Medical Sciences (PIMS), Islamabad.

Bacterial strain

Pseudomonas aeruginosa (ATCC® 51679™) was used in this study. Before using the strain in the assay, bacterial cultures were identified via Gram-staining, oxidase and catalase test as Gram-negative, oxidase and catalase positive.

Stock culture preparation

To prepare a stock culture, one colony of *P. aeruginosa* from the MacConkey agar plate was inoculated into 10 ml of nutrient broth and incubated overnight at 37°C. Next day, 15% of glycerol was mixed with bacterial growth in log phase and stored at –80°C as 300 µl aliquots for future use.

Preparation of sera

After receiving written consent, blood samples were collected from 60 healthy subjects, 20 each from three selected age groups. 10 ml of blood was drawn by venipuncture and transferred to polypropylene tubes (Becton Dickinson). To prevent the complement activation, tubes were immediately transferred to the ice. Serum was separated after 1–3 hours of incubation on ice by centrifuging the blood for 7 minutes at 7000 rpm. A portion of each sample was heat inactivated at 56°C for half an hour to inactivate the complement system. Serum was stored in a –80°C freezer until used in experiments. Ethical approval for the collection of blood was provided by the ethical committee of PIMS, Islamabad.

Serum bactericidal assay

An aliquot of bacteria was centrifuged at 10,000 rpm for 2 minutes and the pellet was washed by resuspending in 300 ml of Hanks' Balanced Salt Solution (HBSS) (Sigma-Aldrich). After centrifugation, the bacterial pellet was subsequently diluted in HBSS to achieve a bacterial count of 1×10^8 CFU/ml. The SBA for each serum sample was carried out in two Eppendorf tubes, one for normal human serum and the other for heat inactivated serum as a negative control. The total volume of each tube was 1 ml, which contained 10% of serum and 1×10^8 CFU/ml of bacteria suspended in HBSS solution. Both tubes were incubated for two hours at 37°C in a rotary mixer. During incubation, 20 ml of the sample was separated from each tube at time points 0, 30, 60, 90 and 120 minutes and diluted in saline at different concentrations. Selected time points were chosen to evaluate the progress/response time of sera from selected age groups in killing *P. aeruginosa* during the experiment. 20 ml of each dilution was spread onto MacConkey agar plates and incubated overnight. Colonies were counted next day to determine the CFU/ml of each sample.

Statistical analysis

Data were analyzed by using GraphPad Prism, Version 6 (GraphPad Software). The significance of differences in means of different groups was determined by using Student's unpaired *t*-test. Correlation between age and killing activity was analyzed by the third order polynomial (cubic), drawing an interpolated standard curve. The differences were considered significant when a p value of < 0.05 was obtained.

Results

Bactericidal activity of fresh human sera and heat-inactivated sera

Bactericidal activity of sera, collected from three selected age groups, was investigated in this study. Two types of sera from each individual were examined; the normal human serum and the heat inactivated serum in which the complement was deactivated and used as a negative control. Both the fresh and heat inactivated sera collected from children were completely compromised in their ability to kill *P. aeruginosa* (Fig. 1A). In adults, fresh sera exhibited a significantly higher level of killing of *P. aeruginosa* as compared to heat-inactivated sera with p values of < 0.0001, 0.0008 and < 0.0001 at time points 60, 90 and 120 min during incubation (Fig. 1B). Similarly, fresh sera from elderly also showed a significantly higher level of killing as compared to heat-inactivated sera with p values of 0.0003, 0.0067 and < 0.0001 at time points 60, 90 and 120 min, respectively (Fig. 1C).

Comparison of bactericidal activity of normal human sera of different age groups

When a comparison in killing of *P. aeruginosa* by fresh serum collected from three age groups was made, it was evident that adult sera were highly efficient in the killing of *P. aeruginosa* with substantially decreased mean CFU/ml at time points 90 and 120 minutes, whereas children sera did not show any decrease (Fig. 2). Bacterial killing of adult sera was significantly higher as compared to children with p values of < 0.0001, 0.002 and < 0.0001 at time points 60, 90 and 120 min, respectively. On the other hand, elderly sera also exhibited a significantly higher level of bactericidal activity as compared to children after 60, 90 and 120 min with p values of < 0.0001, 0.0036 and < 0.0001, respectively. Although, the killing of elderly sera was decreased to a certain degree as compared to adults, statistical analysis did not affirm any significant differences between these two age groups (Fig. 2).

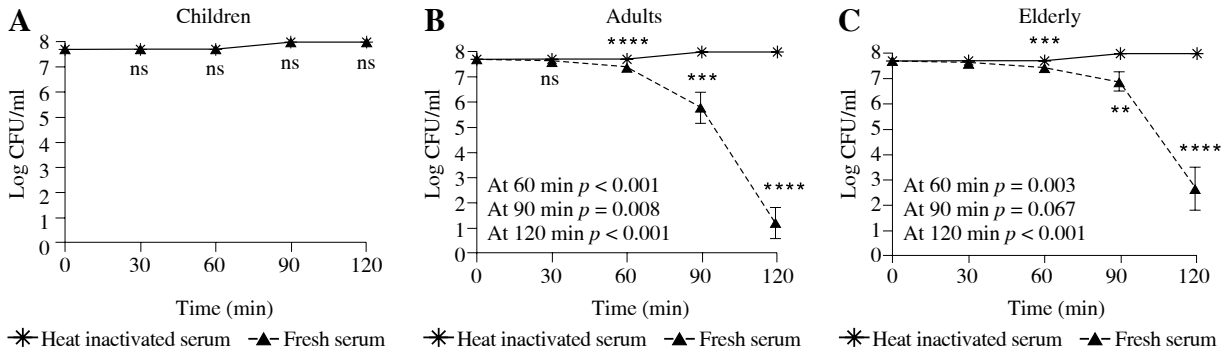

Fig. 1. Viable *Pseudomonas aeruginosa* counted after incubation with fresh human sera (filled triangles) and heat inactivated sera (crosses) collected from children (**A**), adults (**B**) and elderly (**C**). Samples were removed from *P. aeruginosa*/serum mixture at selected time points and CFU/ml determined. Fresh human serum and heat-inactivated serum from each individual were run in parallel. Each value indicates mean (±SEM) of the log CFU/ml of twenty samples from respective age group. Statistical differences between means were analyzed by Student's unpaired *t*-test

Correlation between age and *Pseudomonas aeruginosa* killing

Analysis of all 60 volunteers showed a significant correlation between age and bactericidal killing of *P. aeruginosa*. A scattered graph of all samples revealed that all the sera of children under 10 years did not kill bacteria, and the bacterial count remained similar even after the incubation of 120 min. When the age exceeded ten, sera revealed some killing with the increasing age. Sera from individuals between 18 and 45 were highly efficient in killing *P. aeruginosa*, a majority of which completely killed bacteria by time point 120. When age exceeded 45 years, the bulk of sera showed a decline in the killing. Only three sera from this group completely killed bacteria, whereas rest exhibited reduced killing. As obvious from scatter graphs that the overall trend in the bacterial killing was an initial increase from children to adults and a subsequent decrease from adults to elderly. Therefore, to determine the correlation between age and *P. aeruginosa* killing, data were analyzed by the third order polynomial (cubic) after drawing an interpolated standard curve. Results suggest a significant correlation between age and bactericidal killing with coefficient of determination values of 0.34, 0.27 and 0.58 and p values of < 0.0001, < 0.001 and < 0.0001 at time points 60, 90 and 120 min, respectively (Fig. 3).

Discussion

The serum is an unfavorable environment for invading pathogens due to its bactericidal properties mediated by a system of more than 35 proteins collectively known as the complement system. This phenomenon has been widely known since the late 19th century. Bactericidal effects of serum can be demonstrated *in vitro* against bacteria, especially Gram-negative pathogens [34–36]. Various studies have documented the bactericidal activity of serum against *P. aeruginosa* to investigate different aspects of host interaction with *P. aeruginosa* [23, 26, 37, 38]. Here we described the age-related differences in the serum-killing of *P. aeruginosa*. Subjects of the study were categorized into three age groups; children, adults and elderly.

The initial evaluation of fresh normal human serum with heat-inactivated serum revealed that all the heat

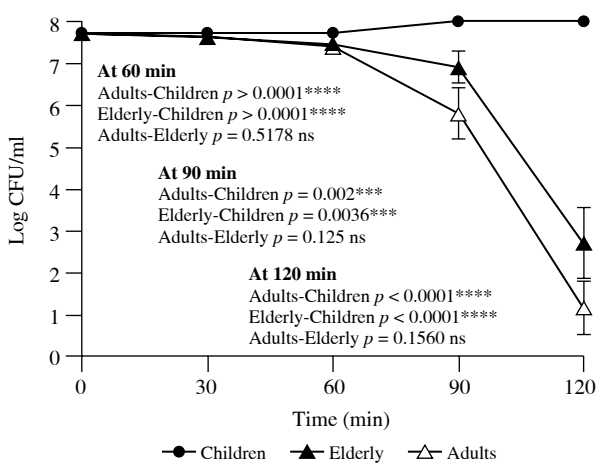

Fig. 2. Comparison between the bactericidal activity of fresh human sera collected from selected age groups against *Pseudomonas aeruginosa*. The highest level of killing was exhibited by adult sera (open triangles) followed by elderly (closed triangles), which was slightly lower than adults. SBA in children (closed circles) was significantly compromised in children sera as compared to adult and elderly sera. Each value indicates mean (±SEM) of the log CFU/ml of twenty samples from respective age group. Statistical differences between means were analyzed by Student's unpaired *t*-test

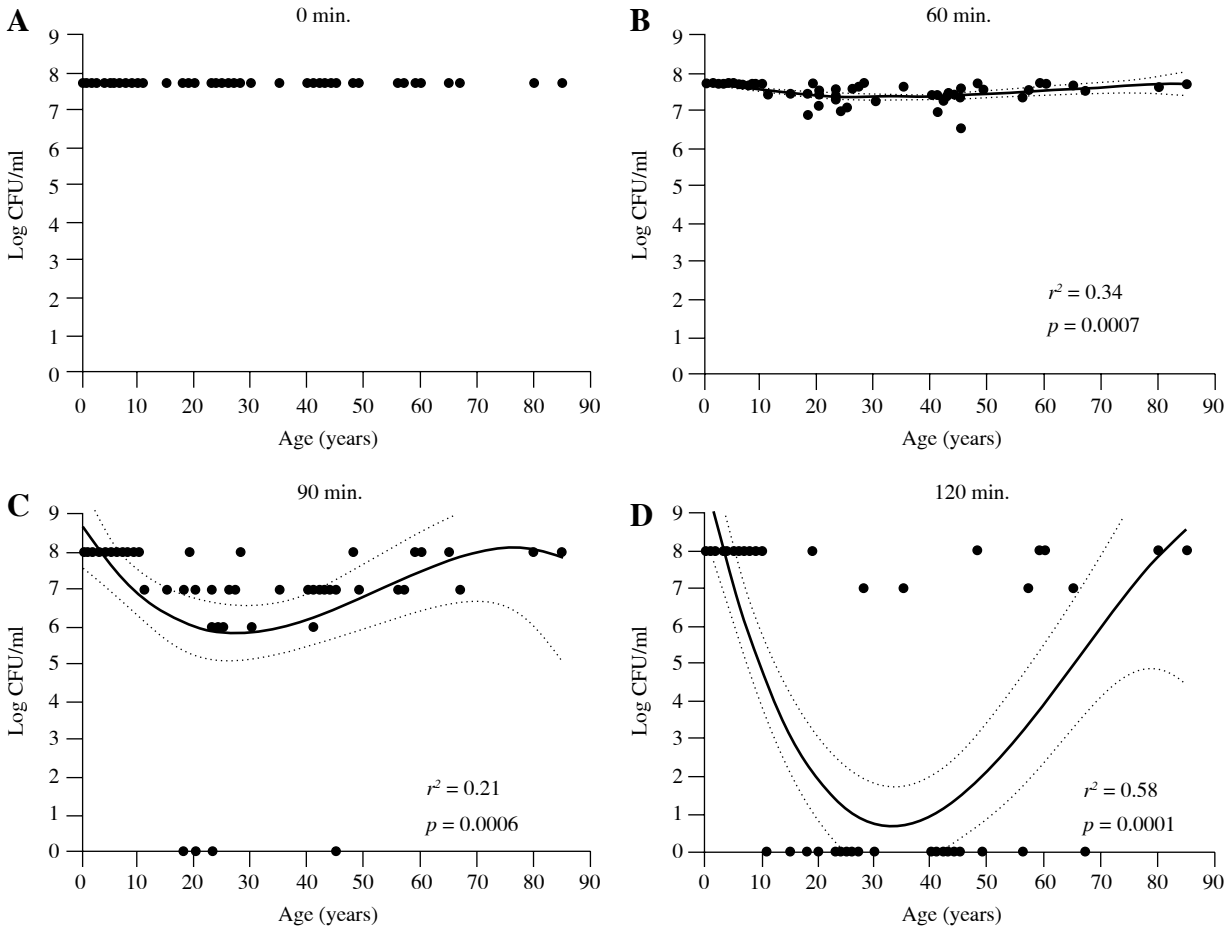

Fig. 3. Scattered-dot plots of CFU/ml of *Pseudomonas aeruginosa* after SBA to determine the correlation of age with the bactericidal killing of serum. Each dot represents the CFU/ml of each individual examined in this study after incubation of bacteria with serum. Each graph represents the viable count of *P. aeruginosa* at selected time point. Solid lines in each graph denote an interpolated curve analyzed by third order polynomial, cubic; dashed lines, 95% CI thereof. Analysis revealed that age is positively correlated with the bactericidal activity of sera

inactivated sera from selected age groups were unable to kill *P. aeruginosa* with no decrease in CFU/ml at all during two-hour incubation. It is a known fact that complement proteins are heat labile, which are deteriorated at high temperatures [39, 40]. On the other hand, fresh sera from adults and elderly individuals showed a significantly higher bactericidal effect against *P. aeruginosa*. Therefore, the absence of bactericidal activity in heat inactivated sera, in line with a higher level of killing demonstrated by fresh human sera, further supports the evidence that bactericidal activity of serum is complement-driven as reported previously by several other authors [16, 22, 41].

Interestingly, fresh sera from children were fully compromised in their ability to kill *P. aeruginosa* with no noticeable differences as compared to heat-inactivated children sera. This is in agreement with a previously reported study documenting a less efficient bactericidal activity exhibited by neonatal sera against several Gram-negative bacteria as compared to normal adult sera [42]. The major contributor of serum bactericidal killing in the absence of blood cell types is the formation of MAC (C5b6789) [21, 43]. Children and neonates have a maturational deficiency of several complement factors [44]. However, the most relevant deficiency that may contribute to the lack of bactericidal activity of children is the C9 and C8 deficiency as described previously, with C9 present in the lowest relative concentration as compared to adults [45, 46]. Although, C8 and C9 might be important in forming MAC, the role of recognition molecules cannot be ignored due to the fact that the complement cannot be activated in the absence of these molecules, and other complement factors become irrelevant if the complement is not activated by these molecules. Different studies have reported lower levels of complement recognition molecules in neonates

and children, which are below normal adult values. These include C1q [47], MBL [48–50], M-ficolin and L-ficolin [50, 51]. Hence, the diminished serum concentrations of MAC components C8 and C9, in parallel with lower concentrations of the recognition molecules for complement activation may explain the inefficiency of children sera in killing *P. aeruginosa*. However, there is a need to precisely investigate the role of these molecules in fighting *Pseudomonas* infection in order to identify the components of the complement system; deficiency of which significantly alters the bactericidal activity in children and neonates against *P. aeruginosa*.

Although, having no significant differences statistically, elderly sera were partially compromised in bactericidal activity against *P. aeruginosa* as compared to fresh adult sera. Most of the serum samples from adults completely killed bacteria by 120 min after incubation. In contrast, only three serum samples from individuals > 45 years totally cleared bacteria by the time point 120 min, whereas the rest of sera from this group revealed partial killing. This is a clear indication that with the age exceeding 45 years, bactericidal activity declines (Fig. 3), which may be due to age-related regression of the complement system. With aging, the immune system undergoes several alterations and remodeling at organic and cellular levels, eventually predisposing elderly to an increased risk of several infections [52, 53]. As a result of these age-related changes, mortality rates in elderly patients are three times higher as compared to adults [54]. Although, the majority of studies have largely focused on defects in the immune system arising with aging, like compromised phagocytosis and cytokine responses [53, 55], age-related dysregulations in the complement system have been largely ignored in humans. However, some animal studies conducted in mice are in agreement with our findings. Hazlett *et al*. reported compromised phagocytosis response in aged mice, arising due to defects in complement response against *P. aeruginosa* [30]. Similarly, other studies also revealed altered immune responses in aged mice against *P. aeruginosa* [56, 57]. Since, the compromised function of the complement system affects several other biological functions like opsonophagocytosis and inflammation, defects in the phagocytosis and other cellular responses in aged mice as described previously, indirectly supports the finding of our study.

In conclusion, *in vitro* findings of our study demonstrate the variations in the bactericidal activity of serum by establishing that children sera show a complete failure, whereas elderly sera display a partial decrease in the killing of *P. aeruginosa*. This indicates that components of the innate immune system, especially the complement system, do not work with full potential in these two age groups as compared to adult sera. Hence, our findings open a window for future research in investigating these components in relation to *P. aeruginosa* infections with an emphasis on those proteins, which are essential in providing defense against *P. aeruginosa*, and are not fully functional in children or partially compromised in elderly. If these innate immune components are successfully identified, they can be reconstituted in children and elderly to compensate the compromised immunity against *P. aeruginosa*, or even boost the innate immune response in adults. Immune therapy strategies like these may be a substitute for 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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The authors declare no conflict of interests.

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