

# Induction of innate immune responses by KPC-producing *Klebsiella pneumoniae* of the pandemic sequence type 258-clade I

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## Abstract

*Klebsiella pneumoniae*-carbapenemase-producing *K. pneumoniae* (KPC) sequence-type 258 (ST258) has emerged as an important human pathogen throughout the world. Although lacking known virulence factors, it is associated with significant morbidity and high mortality rates. The pathogenicity of KPC *K. pneumoniae* ST258 strains has not been fully elucidated yet. We sought to investigate the interactions of the KPC *K. pneumoniae* ST258-clade I with different components of innate immunity. Human serum was used to evaluate the serum bactericidal activity and the J774A.1 murine (BALB/c mice) macrophage cell-line was used to examine phagocytosis, mRNA expression and production of the pro-inflammatory cytokines IL-1 $\beta$ , TNF- $\alpha$  and IL-6. L-78, a KPC-producing *K. pneumoniae* ST258-clade I strain was used as representative of the strains circulating in Greek hospitals. *K. pneumoniae* ATCC 43816, a virulent K2 strain, was used for comparison. Strain L-78 was susceptible to human serum and rapidly phagocytosed by J774A.1 cells, in contrast to the virulent K2 strain, which was serum-resistant and slowly phagocytosed. Stimulation of the J774A.1 cells with the L-78 strain induced production of IL-1 $\beta$  at concentration levels significantly higher compared to K2, whereas production of TNF- $\alpha$  and IL-6 levels were comparable by the two strains. L-78 was able to induce IL-1 $\beta$  mRNA and NLRP3 mRNA expression. Our findings indicate that *K. pneumoniae* ST258-clade I is serum sensitive, rapidly phagocytosed and is capable of eliciting adequate innate immune response in terms of production of pro-inflammatory cytokines.

## INTRODUCTION

The carbapenem resistance in *Klebsiella pneumoniae* (Kp) is driven primarily by isolates that are members of the 258 clonal complex (CC258), with sequence-type 258 (ST258) being predominant producing either the KPC-2 or the KPC-3 carbapenemases [1, 2]. ST258 strains are endemic in many parts of the world and comprise two distinct genetic ST258 clades, I and II [3]. These strains can cause severe infections associated with increased morbidity and high mortality rates [4]. It could be hypothesized that ST258-Kp strains are endowed with virulence traits that may aid their ability to evade the immune response and affect infection

outcomes. This hypothesis has been supported by recent experimental findings suggesting that bacteria of ST258 clade I may partially escape innate immune recognition and IL-1 $\beta$  mediated inflammation [5]. In different experimental conditions however, using a mouse septicemia model, it was demonstrated that *K. pneumoniae* (capsular serotype K41, KPC-2-producing) ST258 clade I strain, representative of those circulating in Greek hospitals, exhibited low virulence in comparison with a highly virulent capsular serotype K2 strain [6]. We sought to contribute further to this discussion by examining the interactions of a ST258 clade I strain with different components of innate immunity and compare the

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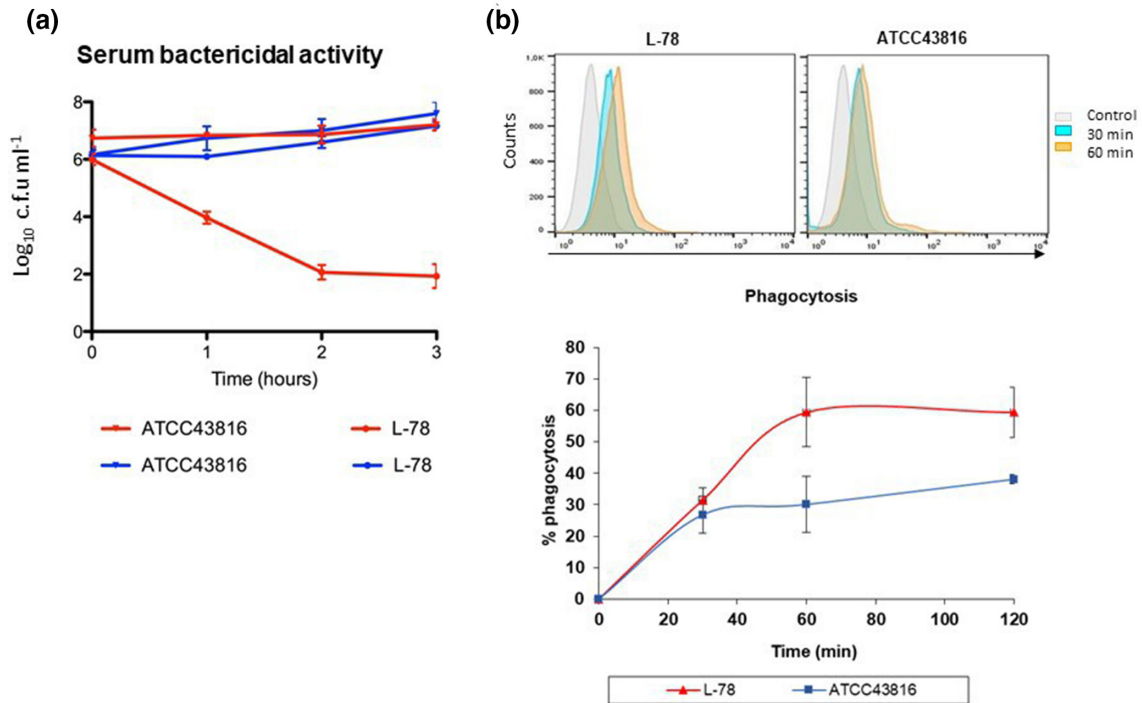
**Keywords:** IL-1 $\beta$ ; KPC-producing ST258; *Klebsiella pneumoniae*; NLRP3; pro-inflammatory cytokines.

**Abbreviations:** ATCC, American Type Culture Collection; CFU, colony-forming units; DMEM, Dulbecco's Modified Eagle Medium; DPBS, Dulbecco's phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1 $\beta$ , interleukin-1 beta; IL-6, interleukin-6; KPC, *Klebsiella pneumoniae*-carbapenemase; LPS, lipopolysaccharide; NLRP, NOD-, LRR- and pyrin domain-containing protein; PBMCs, peripheral blood mononuclear cells; PFGE, pulsed-field gel electrophoresis; RT-PCR, real-time PCR; ST258, sequence type 258; TNF- $\alpha$ , tumor necrosis factor-alpha.

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**Fig. 1.** Bactericidal activity of human serum (red lines) and heat-inactivated serum (blue lines) against L-78 (KPC-producing ST258 clade I) and reference strain ATCC 43816; each point represents the mean of  $\log_{10}$  c.f.u.  $\text{ml}^{-1} \pm \text{SD}$  of three different experiments (a). Phagocytosis of strain L-78 and reference strain ATCC 43816 by macrophages of the J774A.1 murine (BALB/c mice) cell-line (b). Representative fluorescence-activated cell-sorting histogram plots (upper panel) and phagocytosis rates of L-78 and ATCC43816 by J774A.1 (lower panel). In the upper panel, grey histograms represent J774A.1 cells in the absence of bacteria (negative control). Light blue and orange histograms represent cells in the presence of bacteria at 30 and 60 min time-points, respectively. In the lower panel, results are expressed as the percentage (%) of macrophages that internalized bacteria (% phagocytosis) versus time. Results are the means of three independent experiments  $\pm \text{SD}$ .

results with those obtained with the known virulent *K. pneumoniae* ATCC 43816 strain.

## METHODS

### Strains

The *K. pneumoniae* L-78 strain (capsular type 41) was used as representative of KPC-2 producing ST258 clade I. This strain was from our collection of 110 well-characterized (STs, PFGE types, plasmid content,  $\beta$ -lactamase production and resistance phenotypes) KPC-Kp ST258 isolates, derived at random from bacteremia patients in Greek hospitals during 2009 to 2011 [6].

*K. pneumoniae* ATCC 43816 sequence-type 439 (ST439), a highly virulent capsular serotype K2 strain commonly used in animal infection models, was used as the reference strain for comparison.

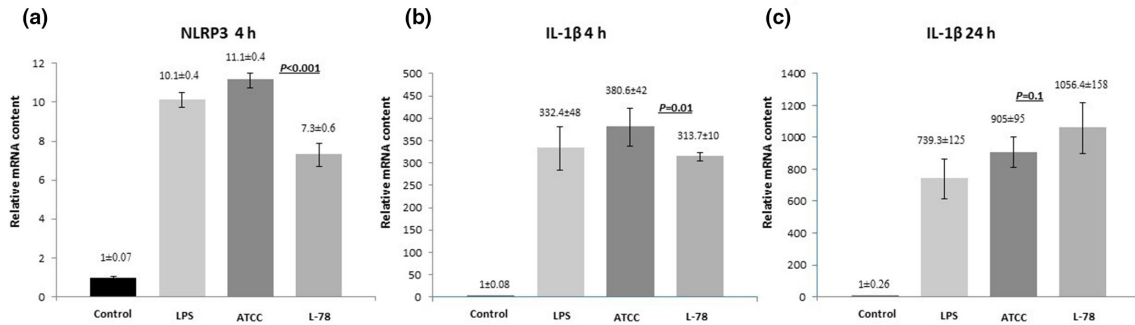
### Serum bactericidal activity assay

Susceptibility to human serum was assessed using the method of Hughes *et al.* [7]. Briefly, serum was obtained from five healthy volunteers on the day of each experiment. A volume of 0.5 ml inoculum of early log-phase bacterial cells at a

concentration of  $6 \times 10^6$  c.f.u.  $\text{ml}^{-1}$  was added to 1.5 ml of undiluted serum and incubated at  $37^\circ\text{C}$  for 24 h. Aliquots were obtained at 0, 1, 2, 3 and 24 h and serial tenfold dilutions were plated onto Müller–Hinton agar plates. After a 24 h incubation at  $37^\circ\text{C}$ , c.f.u. of each sample were counted. Similar experiments with heat-inactivated serum at  $56^\circ\text{C}$  for 30 min were also performed. Each experiment was performed three times, and the results were expressed as the means of  $\log_{10}$  c.f.u.  $\text{ml}^{-1}$ . Responses were interpreted as previously described [7]. More specifically, they were graded from 1 to 6 according to viable counts (VC) expressed as percentage of the inoculum. Grades 5 and 6 are considered resistant; grades 1 and 2 susceptible and grades 3 and 4 intermediate.

### Phagocytosis assay

Phagocytosis was performed using the J774A.1 murine (BALB/c mice) macrophage cell-line (LGC Promochem), as described previously [6, 8]. Briefly, the J774A.1 cells were grown in DMEM with 10% foetal bovine serum (FBS) with penicillin/streptomycin. Subsequently, the cells were grown overnight in a 24-well plate at a confluence of  $5 \times 10^5$  cells in DMEM-1% FBS-without antibiotics. Adherent macrophages were then washed with prewarmed Dulbecco's PBS (DPBS),



**Fig. 2.** Gene expression of the NLRP3 inflammasome and IL-1 $\beta$  by macrophages (J774 murine cell-line) after stimulation with L-78, reference strain ATCC 43816 and LPS, determined by RT-PCR: NLRP3 at 4 h (a), IL-1 $\beta$  at 4 h (b), IL-1 $\beta$  at 24 h of stimulation (c). Results are presented as mRNA content relative to unstimulated cells and normalized to glyceraldehyde-3-phosphate dehydrogenase GAPDH expression. Values are expressed as the mean  $\pm$  SE of four independent experiments measured in duplicate.

and DMEM without FBS and antibiotics were added prior to the addition of labelled bacterial cells. The bacterial cells (L-78 and ATCC43816) were labelled with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Invitrogen, USA) according to the manufacturer's instruction. The medium was replaced with ice-cold DMEM containing the labelled bacteria at a m.o.i. of 50. The macrophages were placed on ice for 30 min to allow the synchronization of bacteria binding onto the cell and then incubated at 37°C for 15, 30, 60 and 120 min. Phagocytosis was stopped by adding of ice-cold 5 mM DPBS/EDTA to each well, cells were washed three times with DPBS/EDTA and once with ice cold DPBS in order to remove non-internalized bacteria attached to the cell surface. Internalization of bacteria was monitored by flow cytometry with a BD FACSCalibur (Becton Dickinson, CA, USA) for 2 h. For each sample 30.000 events were acquired in flow cytometer and fluorescent cells were gated against non-fluorescent cells of negative control. Experiments were performed in triplicates and data were analysed using FlowJo software version 10.0 (Tree Star Inc., Ashland, OR, USA). The results are presented as the percentage of cells having internalized bacteria (% phagocytosis).

## Cytokine production

### Stimulation of macrophages *in vitro*

Strains L-78 and ATCC 43816 were grown to log-phase in Müller-Hinton broth at 37°C. A volume of 3 ml of each culture was centrifuged, reconstituted in 1 ml PBS, and inactivated in water bath at 65°C for 30 min. After a second centrifugation and reconstitution in 1 ml PBS, bacteria were stored at -80°C until use.

J774A.1 cells were cultured in DMEM (high-glucose Dulbecco's Modified Eagle Medium) (Gibco, Invitrogen, USA) supplemented with L-glutamate, 1% penicillin/streptomycin and 10% FBS (Gibco, Invitrogen, USA), and incubated at 37°C in 5% CO<sub>2</sub> atmosphere. Macrophages were subsequently cultured on 12-well plates at a final concentration of 10<sup>6</sup> cells ml<sup>-1</sup> and stimulated with heat-inactivated bacteria (L-78, ATCC 43816, at a 1:1 ratio), and LPS (1  $\mu$ g ml<sup>-1</sup>) (*E.*

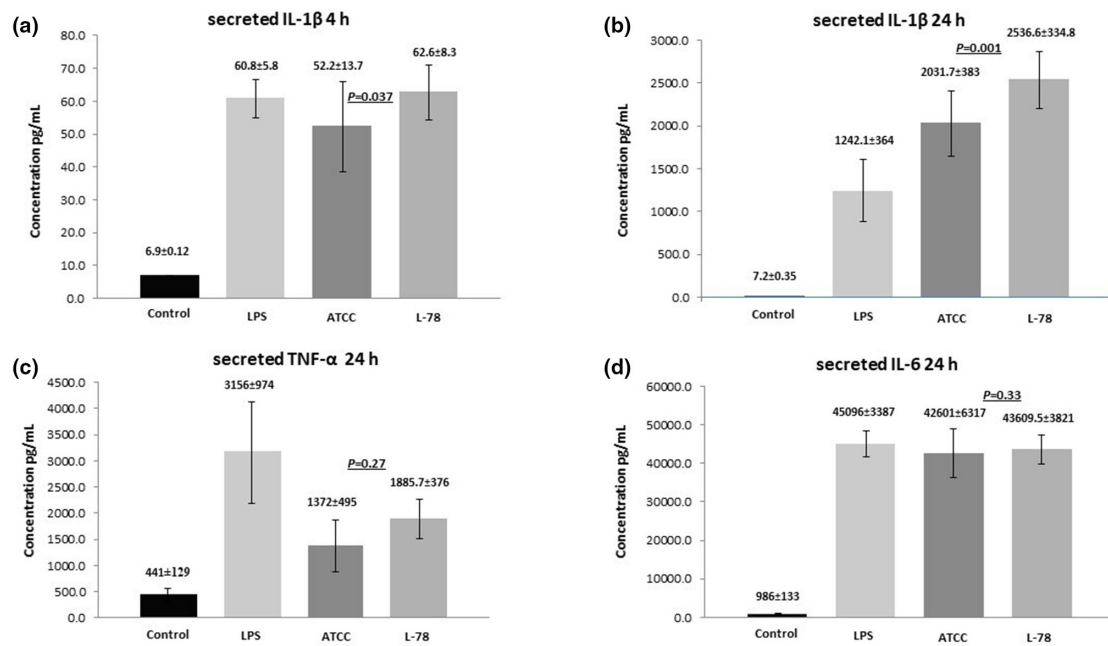
*coli*, purified Sigma-Aldrich, Germany). Experiments were performed in triplicate and repeated two times. Untreated cells were used as the control. At 4 h and 24 h the cells were harvested in lysis buffer and used for mRNA gene-expression analysis whereas supernatants were removed and used for pro-inflammatory cytokine quantification.

### Quantification of cytokines

Production of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  from culture supernatants was quantified by an ELISA using a High Sensitivity Bead-Based Multiplex Assay kit HSTCMAG-28SK and the Luminex technology (EMD Millipore, MILLIPLEX) following the manufacturer's instructions. No dilution of the supernatants was required.

### mRNA gene expression

After stimulation of the macrophages with L-78, ATCC 43816 and LPS, RNA was extracted from J774A.1 cells with Nucleospin RNA Plus (Macherey Nagel, Germany). The quality of RNA was assessed by electrophoresis on 2% agarose gel and the quantity was measured by spectrophotometry at 260 nm. Reverse transcription was performed using 1  $\mu$ g quality-controlled RNA and the iScript cDNA synthesis kit (BIORAD, USA). A quantitative real-time PCR (RT-PCR) was used to measure the levels of expression of IL-1 $\beta$  mRNA and the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome mRNA, with Taqman probes (ThermoFisher, USA). Search information obtained from GenBank was used in order to design the primers spanning two different exons (ThermoFisher, <https://www.thermoFisher.com/gr/en/home/life-science/oligonucleotides-primers-probes-genes/applied-biosystems-custom-primers-probes.html>). The Gene Accession numbers and Taqman code numbers were: IL-1 $\beta$ ; BC011437.1, Mm00434228\_m1 (PCR product 90 bp, exons 3–4), NLRP3; NM\_145827.3, Mm00840904\_m1, (PCR product 84 bp, exons 2–3) and GAPDH; NM\_008084.3, Mm99999915\_g1 (PCR product 107 bp, exons 2–3), respectively.



**Fig. 3.** Production of cytokines by macrophages quantified in culture supernatants after stimulation with heat-inactivated bacteria (L-78, ATCC 43816) and LPS: IL-1 $\beta$  at 4 h (a), IL-1 $\beta$  at 24 h (b), TNF- $\alpha$  at 24 h (c), IL-6 at 24 h (d). Results are expressed as concentration in pg ml<sup>-1</sup>, mean values $\pm$ SE of four independent experiments.

The mRNA levels were calculated relative to the internal control GAPDH (glyceraldehyde-3-phosphate dehydrogenase) house-keeping gene, and the relative amount of mRNA was calculated by using the  $2^{-\Delta\text{CT}}$  method. Four independent experiments were performed.

### Statistical analysis

Student's *t*-test was applied and a *P*-value of  $\leq 0.05$  was considered significant.

## RESULTS AND DISCUSSION

The pathogenicity of KPC *K. pneumoniae* ST258 strains has not been extensively investigated. Several virulence factors have been acknowledged and various assays and infection models have been employed in order to elucidate the mechanisms behind the significant morbidity and mortality rates [6, 9, 10]. The presence of KPC itself does not appear to correlate strongly with the virulence of ST258 or other *K. pneumoniae* strains [11, 12], and unlike the hypervirulent *K. pneumoniae* strains (capsular serotype K1 and K2) [13], the ST258 bacteria affect mainly hospitalized patients with severe underlying diseases and comorbidities, acting merely as opportunistic pathogens [6, 14, 15]. Herein we studied the innate immune response elicited by a representative strain of ST258 *K. pneumoniae* of clade I (L-78) and the results were compared to those obtained by a hypervirulent K2 reference strain (ATCC 43816).

The L-78 strain was killed by human serum rapidly (4  $\Delta\log_{10}$  c.f.u. reduction within 3 h). Apparently, bacterial killing

was complement-mediated, as serum bactericidal activity was abolished after heat-inactivation of the serum (Fig. 1a). Unlike the L-78, the ATCC 43816 strain was serum-resistant, as its viable counts remained unaffected after a 3 h incubation period, thus confirming our previous results [6]. At 24 h the K2 strain had full growth ( $5 \times 10^9$  c.f.u. ml<sup>-1</sup>) whereas no viable counts were detected for the L-78 strain (data not shown). Differences between the two strains were also observed in phagocytosis experiments; L-78 was internalized at high rates (60% within 60 min) whereas only 30% of ATCC 43816 was phagocytosed in the same time period as shown in Fig. 1b, also confirming previous results [6]. Similar observations were made by Chiang *et al.* using three different ST258 clinical strains, from Taiwan and the USA, in a phagocytosis assay with human neutrophils [11]. It has to be noted however, that in our experiments only the internalization of bacteria by macrophages was examined and not the interactions between bacteria and effector cells after engulfment. Cano *et al.* [16] elaborated further on the interactions between *K. pneumoniae* and macrophages *in vivo*, in mice infected intranasally with the hypervirulent 43816 *K. pneumoniae* strain. They showed that this strain survived killing by macrophages for hours, by impairing phagosome maturation and phago-lysosome fusion. Furthermore, they showed that this strain triggered a programmed cell death in macrophages displaying features of apoptosis [16]. It is not known whether ST258 clade I can impair phagosome maturation or if it is able to trigger apoptosis of macrophages.

It has been suggested that changes in capsular synthesis may help some strains evade primary immunity. Castronovo *et al.*

[5] showed that ST258 clade I and clade II differed in their ability to activate monocytes and myeloid dendritic cells from human immune competent hosts. The induced inflammatory response was more pronounced for clade II compared to clade I, suggesting that clade I may partially escape innate immune recognition and IL-1 $\beta$ -mediated inflammation. To gain further information regarding the interactions of clade I with innate immunity we investigated the effect of L-78 on the expression of NLRP3 and IL-1 $\beta$  genes compared to ATCC 43816. IL-1 $\beta$  is an important mediator of inflammation, having a central role on the influx of inflammatory cells, but having also a role in the adaptive and trained immunity [17]. Pattern recognition receptors (PRRs) and cytokine receptors control the transcription of the pro-IL-1 $\beta$ , whereas its proteolytic processing to the active mature IL-1 $\beta$  form and release into the extracellular space is regulated by the NLRP3 inflammasome protein complex [18, 19]. As presented in Fig. 2(a, b), bacterial cells of L-78 were able to activate the transcription of NLRP3 and IL-1 $\beta$  genes, although less efficiently compared to ATCC 43816 when examined at 4 h of incubation. At 24 h however, the difference of IL-1 $\beta$  gene expression between the two strains was abolished (Fig. 2c). A study utilizing the ATCC 43816 *K. pneumoniae* strain as a model organism, has shown that NLRP3 is a critical regulator of *K. pneumoniae* induced inflammation and cell death [20]. More recently, Castronovo *et al.* showed that purified capsular polysaccharides from ST258-KP bacterial cells affected the NLRP3 (but not the NLRP4) inflammasome and the pro-IL-1 $\beta$  gene expression, through the p38MAPK- and NF- $\kappa$ B protein signalling pathways [5]. Although not designed to explore the activation of NLRP3, our study indicates that the NLRP3 inflammasome pathway has been involved in the release of IL-1 $\beta$  upon stimulation with our strains.

In parallel experiments we examined the ability of the two strains to induce production of pro-inflammatory cytokines. At 4 h after stimulation, the concentrations of IL-1 $\beta$  were similar for both strains (Fig. 3a), despite the lower IL-1 $\beta$  gene expression found for L-78 at this time-point. At 24 h (Fig. 3b) the total amount of secreted IL-1 $\beta$  after stimulation with L-78 increased further, at levels significantly higher compared to ATCC 43816 ( $P=0.001$ ).

Production of TNF- $\alpha$  was also higher after stimulation with L-78 compared to ATCC 43816 (Fig. 3c) but not significantly. The ability of the two strains to induce production of IL-6 was similar (Fig. 3d).

These results are in agreement with findings of others using human peripheral blood mononuclear cells (PBMCs) as effectors [5, 21], though quantitative differences between strains may exist. In the present work, we showed that the production of IL-1 $\beta$  after stimulation with a representative strain of clade I was more pronounced compared to a virulent K2 strain. Further research will be needed to show whether these increased levels have any clinical significance.

In line with previous observations, the activation of the inflammasome through the NLRP3 pathway may play a central role for the production of IL-1 $\beta$  by ST258-clade I

[5, 20]. Apparently, the activation and employment of the cellular effectors and mediators mentioned above will help the host to contain infection and mediate clearance of bacteria. It should be noted however, that the results presented herein were observed in strictly controlled experimental conditions and their significance cannot be extrapolated to the much more complex human defence system and especially in the immunocompromised. Overgrowth of these bacteria due to inadequate therapies may overwhelm the already impaired immune system of the severely ill or other vulnerable patient categories, as previously suggested [6, 22]. In these patients pro-inflammatory cytokine induction evidently depends also on the state of the effector cells. Further work needs to be done to confirm our findings and unravel why these organisms are associated with high mortality rates and why they have the ability to disseminate and persist in humans.

A limitation of this study is the use of a single strain of ST258. However, L-78 was chosen as a well-characterized clade I strain that had been used previously by our team also *in vivo*, in a mouse septicemia model, showing very low virulence [6]. In fact, L-78 was unable to kill immunocompetent animals even when they were infected with a large inoculum ( $LD_{50} >10^8$  c.f.u.).

In conclusion, the findings presented herein indicated that *K. pneumoniae* ST258-clade I was not able to evade the innate immune response; it was killed rapidly by human serum from healthy volunteers and phagocytosed faster and at higher rates by murine macrophages compared to a K2 virulent strain. Moreover, ST258-clade I was able to induce substantial amounts of pro-inflammatory cytokines IL-1 $\beta$ , IL-6 and TNF- $\alpha$ , that are known to be involved in controlling *K. pneumoniae* infections [9].

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#### Conflicts of interest

The authors declare that there are no conflicts of interest.

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