ORIGINAL RESEARCH

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Pandrug-resistant isolate of *Klebsiella pneumoniae* causes less damage than drug-susceptible isolates in a rabbit model

Abstract

Purpose: Bacterial infections induce a series of inflammatory responses and lead to longer hospital stays and increased mortality. In clinical work, we often find that infections caused by drug-susceptible isolates have a worse outcome than those caused by pandrug-resistant isolates. To goal of this study was to assess the impact of drug resistant in a rabbit model of *Klebsiella pneumoniae* infection.

Methods: This study used a rabbit model of experimental bacteremia, challenged by susceptible (A), multidrug-resistant (B) and pandrug-resistant (C) isolates of *Klebsiella pneumoniae*. Minimal inhibitory concentrations (MIC), leukocyte, TNF α , IL-17, and HMGB-1 levels and survival times were measured.

Results: Mean survival times after challenge by isolates A, B and C were 10.5 ± 3.63 , 12.7 ± 2.31 and 13.9 ± 0.32 days, respectively. Leukocytes levels after challenge with isolate C were lower compared with those after challenge with isolate A ($p = 0.002$). Blood counts of the offending pathogens and concentrations of TNF α , IL-17, and HMGB-1 were higher in the group challenged by isolate A in comparison with isolate B or C. Tissue bacterial load after animal death was significantly higher in rabbits of group A in comparison with isolates B and C.

Conclusion: Bacteremia induced by pandrug-resistant isolates is accompanied by less damage compared with bacteremia by drug-susceptible isolates. Rabbits infected with a pandrug-resistant isolate of *K. pneumoniae* survived longer and had a lower inflammatory response than did animals infected with drug-susceptible isolates.

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Bacterial infections cause mild to life-threatening illnesses through activation of a series of proinflammatory, anti-inflammatory and apoptotic cascades that ultimately result in a disruption of physiologic homeostasis [1-5]. Some studies have concluded that patients infected with bacteria susceptible to drug treatment had a better outcome than did patients infected with multidrug-resistant bacteria [6, 7]. Other studies by Giamarellos-Bourboulis and Bristianou showed that rabbits infected with drug susceptible strains of *E. coli* had worse outcomes than those infected with multidrug-resistant strains, and cytokine release patterns were different [8-10]. Glauser suggested that multidrug-resistant isolates might interfere with the mechanism leading to sepsis, resulting in lower pathogenicity [11]. Recently, due to the development of pandrug-resistance bacterial strains, this controversy has become more complicated; however, there are no clear data regarding the extent to which pandrug-resistance bacteria influences mortality of patients. In order to confirm the differences among susceptible, multidrug-resistant and pandrug-resistance isolates, we established a rabbit model of experimental bacteremia simulating central venous catheter infection caused by *Klebsiella pneumoniae*. Plasma cytokine release and survival following inflammatory response was assessed.

Animals and Methods

Animals

A total of 30 New Zealand rabbits of mean ($\pm$ standard deviation) weight 3.32 ± 0.54 kg were used. Animals were housed in a specific pathogen-free room within the animal care facility, given free access to tap water and standard balanced rabbit chow. Animals were housed under 12-h light/dark cycles. The study was approved by the Institutional Animal Care and Use Committee of Nanjing University and the Principles of Laboratory Animal Care were followed.

Bacterial isolates

Three blood culture isolates of *Klebsiella pneumoniae* were used. One isolate was susceptible (A), one multidrug-resistant (B) and one pandrug-resistant (C). All were derived from patients with nosocomial infections. Pulsed field gel electrophoresis (PFGE) was used to analysis the genetic relatedness of the clinical isolates A, B, and C: little genetic variation among the three isolates was seen. Resistance to two or more groups of antimicrobials with different chemical structure was defined as multidrug-resistant. Resistance to all antimicrobials was defined as pandrug-resistant. Minimal inhibitory concentrations (MICs) were determined according to the Clinical and Labo-

ratory Standards Institute (CLSI) procedures. For the drug susceptible isolate A, MICs to amoxicillin/clavulanate, piperacillin, ceftazidime, ciprofloxacin, amikacin, imipenem and colistin were 4/4, 8, 4, 1, 2, 2 and 1 mg/L, respectively. For the multidrug-resistant isolate B, MICs were 128/4, 256, 64, 2, 64, 4 and 1 mg/L, respectively. For the pandrug-resistant isolate C, MICs were >256/4, >512, >256, >128, >256, >256, and 2 mg/L, respectively. As determined by disk diffusion method, including the Ceftazidime and Ceftazidime-clavulanic acid, isolate A was extended-spectrum beta-lactamase (ESBL) negative and isolates B and C were ESBL positive. The drug-resistance gene of SHV was tested for using polymerase chain reaction (PCR) with the following primers: SHV5' 5' GGG TTA TTC TTA TTT GTC GC 3' and SHV3' 5' TTA GCG TTG CCA GTG CTC 3' (928bp) and this gene was found to be present in isolate B and C.

The bacterial concentration was determined by measuring absorbance at 600 nm and compared to a predetermined standard curve. Bacteria were then diluted to the desired concentration for inoculation.

Bacteremia model

Rabbits were sedated by intramuscular injection of 20 mg/kg of ketamine. Anesthesia was maintained by intravenous administration of 20 mg/kg of lidocaine at 20 min intervals. No analgesics were used, as the operation was brief. A 2 cm-long midline incision was made on the neck to expose the right inner jugular vein [8, 9]. An 18G catheter was inserted and 1×10^8 colony forming units (cfu) per kg of the prepared *K. pneumoniae* isolate was injected, in a volume of 2.0 mL. Just prior to the bacterial injection and at 1, 3, 24, 72 and 120 h after bacterial challenge, 3 mL of blood was taken from an ear vein. Of the blood samples, one mL was used for analysis of leukocyte counts and for determination of tumor necrosis factor-alpha (TNF α), interleukin (IL-17) and high mobility group box 1 (HMGB1) levels. The remaining 2 mL were immediately centrifuged at 2000 rpm for 10 min, serum was collected, frozen and kept at -70 °C until cytokine analysis was performed. The catheter was removed and the incision was ligated. During the experiment, no antibiotic was used. For survival studies, rabbits intravenously inoculated with bacteria were monitored twice daily (morning and late afternoon) for signs of illness. Animals were monitored for 14 days after which all surviving animals were sacrificed. Under sterile conditions, segments from the liver, spleen and lower lobe of the right lung were taken for subsequent bacterial quantitation.

Bacterial count

For organ bacterial counts, a small aliquot of tissue homogenate was serially diluted, plated on blood agar plates and incubated at 37 °C. After 24 h, colonies were counted by operators who were blinded to the different treatment groups. Results are expressed as log₁₀ of cfu per milliliter.

Assay for TNF α , IL-17, and HMGB-1

Plasma levels of TNF α , IL-17, and HMGB-1 were determined by enzyme-linked immunosorbent assay (ELISA) using commercially available kits that are selective for rabbit cytokines (Adlitteram Diagnostic Laboratories, USA). Manufacturer's directions were followed. The minimum detectable concentrations were 0.5 pg/mL for TNF α , one pg/mL for IL-17, and one ng/mL for HMGB-1.

Statistical analysis

Comparisons of serum concentration–time curves of each parameter between animals infected by the three isolates were performed by multiple regression analysis. Comparisons between different times of sampling were performed by one-way analysis of variance. Survival of animals infected by each isolate was estimated by Kaplan–Meier analysis. Comparisons of survival after challenge by each isolate were performed by the log-

rank test. Correlation between the number of viable cells in various organs and survival was performed by linear regression analysis. Probability (*p*) values less than 0.05 were considered significant.

Results

Death occurred in seven rabbits after infection with isolate A (mortality rate 70%); rabbits had a mean survival time of 10.5 ± 3.63 days. Rabbits infected with isolate B had a mortality rate of 30% and the mean survival time was 12.7 ± 2.31 days. The mortality rate of rabbits infected with isolate C was only 10% and these animals had a mean survival time of 13.9 ± 0.32 days (for isolate A vs. C, $p < 0.004$) (Fig. 1).

Leukocytes decreased dramatically at one and 3 h after *K. pneumoniae* injection in all three groups, compared to the value just prior to infection. Rabbits challenged with isolate A clearly had more severe inflammatory reactions (Fig. 2A). At 24 h post-infection, leukocyte levels in rabbits challenged with isolate A were significantly higher than in rabbits challenged with B or C (for isolate A vs. C, $p < 0.002$).

Differences in cytokine levels were also observed among the groups. The significantly higher plasma level of TNF α in group A animals compared to those in groups B and C (*p* values?) likely contributed to the increased mortality of these animals (Fig. 2B). At 1 h post-infection in all three groups,

FIGURE 1. Survival increased after infection with multidrug-resistant (B) and pandrug-resistant isolate (C) compared with susceptible isolate (A).

FIGURE 2. Leukocyte count in blood, release of cytokines TNF α , IL-17 and HMGB-1 and bacterial count in blood by three different isolates of *Klebsiella pneumoniae* induction. Isolate (A): susceptible; Isolate (B): multidrug-resistant; Isolate (C): pandrug-resistant

FIGURE 3. Comparisons of bacterial counts of *Klebsiella pneumoniae* from the liver, lower lobe of the right lung and spleen on death or sacrifice after challenge by three different isolates.

Isolate (A): susceptible; Isolate (B): multidrug-resistant; Isolate (C): pandrug-resistant.

TNF α increased to similar levels; however, over time, the levels in animals infected with isolates B and C were lower than levels in animals infected with isolate A (for isolate A vs. B, $p < 0.001$; for isolate A vs. C, $p < 0.001$). Within one h of infection, significant induction of IL-17 was observed (Fig. 2C). At 3 h, levels of IL-17 peaked. Concentrations of IL-17 in animals infected with isolate A were higher than those infected with isolates B and C (for isolate A vs. B, $p < 0.015$; for isolate A vs. C, $p < 0.001$). Differences in serum IL-17 levels between rabbits challenged by isolates B and C were not significant. Unlike levels of TNF α and IL-17, HMGB-1 levels in plasma did not peak until 24 h post-infection (Fig. 2D). Concentrations of HMGB-1 were lower in animals challenged with isolates B and C than A (for isolate A vs. B, $p < 0.001$; for isolate A vs. C, $p < 0.001$).

To determine if infection with pandrug-resistant bacteria results in a reduced bacterial load compared with infection with a drug-susceptible strain, blood and tissue samples taken from animals either at the time of death or on day 14 were analysed. The results of bacterial blood counts are shown in Fig. 2E. Bacterial blood counts in animals infected with isolates A and B were significantly higher than those of animals infected with isolate C (for isolate A vs. B, $p < 0.005$; for isolate A vs. C, $p < 0.001$). There was detectable growth of bacterial colonies

from tissue samples taken from some animals in all the three experimental groups; however, there was no detectable growth of bacteria from certain rabbits challenged with isolate C (Fig. 3).

Discussion

The increasing numbers of pandrug-resistant bacterial strains is particularly prevalent in nosocomial infections worldwide. To counteract this worrisome situation, a more thorough understanding of infection resulting from exposure to pandrug-resistant bacterial strains is appropriate. The present study was designed to provide some answers regarding the impact of pandrug-resistant on the host. Three different strains of *K. pneumoniae* were used in the bacteremia model: one isolate that was susceptible to all antimicrobials tested (isolate A), one multidrug-resistant isolate with moderate MIC values (isolate B) and one pandrug-resistant isolate with high MIC values (isolate C). Rabbits infected with the susceptible *K. pneumoniae* isolate (A) had a markedly enhanced leukocyte and cytokine response and higher mortality than rabbits infected with drug-resistant strains (B and C). Animals infected with isolate C had lower mortality and bacterial load than animals in the other two groups. To the best of our knowledge, this is the first

report demonstrating differences in the mechanism of bacteremia by susceptible and pandrug-resistant *K. pneumoniae*.

Leukocytes are an integral component of response to infection. Some studies have found that patients with higher leukopenic or leukemoid responses to infection have worse outcomes than patients that do not mount as intense a response [12, 13]. In our study, after *K. pneumoniae* infection of rabbits, leukocyte levels increased in all three groups and peaked at 24 h. Animals treated with the susceptible isolate had significantly higher leukocyte levels than animals in the other two groups. Thus, the higher virulence of isolate A was associated with an increased inflammatory response.

Plasma levels of TNF α , IL-17, and HMGB-1 were also measured. TNF α is a peptide mediator released by monocytes and macrophages in response to various stimuli, including bacterial lipopolysaccharides [14]. Overproduction of TNF α is associated with a wide range of pathologic conditions [15]. Marcin found that in rats with cecal ligation and puncture (CLP), plasma levels of TNF α (12 ng/ml) accurately predicted mortality within 24 h [16]. In our study, 3 h after infection with the susceptible *K. pneumoniae* isolate, TNF α levels in plasma rose to a higher level than did plasma levels in the other two groups.

IL-17 is a T cell-derived proinflammatory cytokine that is involved in accumulation and activation of neutrophils [17, 18]. IL-17 receptor-deficient mice infected intranasally with *K. pneumoniae* have increased numbers of recoverable bacteria in the lung, increased bacterial dissemination into the spleen, and reduced overall survival [19]. In sepsis triggered by CLP, IL-17 neutralization improves survival and is associated with significant reductions in bacteremia and systemic cytokine production [20]. In our experiment, an increase of plasma IL-17 levels 3 h post-infection was observed, with higher levels in the susceptible isolate group.

HMGB1 is released by endotoxin-activated macrophages/monocytes and functions as a late mediator of lethal endotoxemia and sepsis [21]. Circulating HMGB1 levels are elevated in animals between 16 and 32 h after onset of endotoxemia or sepsis and in patients with sepsis [21, 22] or hemorrhagic shock [23]. *In vitro*, HMGB1 can induce production of TNF and IL-1 by macrophages, monocytes, and neutrophils in a p38 MAPK-dependent mechanism. Plasma HMGB1 levels reached a peak at 24 h, with animals in the drug-susceptible group having higher levels than the animals in the pandrug-resistant groups.

In conclusion, rabbits infected with a pandrug-resistant isolate of *K. pneumoniae* survived longer and had a lower inflammatory response than did animals infected with drug-

susceptible isolates. An explanation of the animal findings appears to be the different growth rate of bacteria in blood and tissues. The second hypothesis is that some antibiotics may induce the loss of virulence factors contained in pathogenicity islands (PAIs) of the prokaryotic genome. Further studies will be required to clarify these findings.

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