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Short Communication

Relationships between phagocytosis, mucoid phenotype, and genetic characteristics of *Klebsiella pneumoniae* clinical isolates

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Abstract

Introduction: The relationships between phagocytosis, and mucoid phenotype, plasmid profile and virulence, and resistance genetic characteristics of *Klebsiella pneumoniae* clinical isolates were evaluated. **Methods**: Thirty isolates were used to determine the mucoid aspect. Four were selected for analysis of phagocytosis by alveolar macrophages. **Results**: Thirty percent of the samples presented the mucoid phenotype. The phagocytosis rate ranged from 21.5% to 43.43%. Phagocytosis was not correlated with the plasmid profile, but was apparently correlated with mucoid phenotype and antibiotic susceptibility. **Conclusions:** Several virulence factors act in parallel in *K. pneumoniae* to impair host defense.

Keywords: *Klebsiella pneumoniae*. Alveolar macrophages. Phagocytosis. Mucoid phenotype. Genetic characteristics.

Klebsiella pneumoniae is an enterobacterium responsible for urinary tract infections, pneumonia, meningitis, septicemia, and wound infections, especially in hospitalized immunocompromised individuals[1]. The bacteria tend to produce extended-spectrum β-lactamases (ESBLs), carbapenemase (*K.pneumoniae* carbapenemase, KPC), and efflux pumps[1,2,3].

The many virulence factors in *K. pneumoniae* that determine bacterial pathogenicity include the polysaccharide capsule, which constitutes a physical barrier and hinders phagocytosis[4]. Other factors include adherence to host cells via fimbrial and non-fimbrial adhesins, synthesis of siderophores (yersiniabactin, enterobactin, and/or aerobactin)[4] allowing uptake of iron, the presence of lipopolysaccharide to protect bacteria from the bactericidal effect of antibodies and complement system, and the mucoid phenotype of colonies[5]. The latter pathogenicity factor of *K. pneumoniae* is associated with the presence of a 180 kb plasmid[6,7].

Immunological studies have demonstrated the role of macrophages in the immune system, emphasizing the importance of analyzing the virulence of bacterial strains and the response developed by the host[8,9]. One of the defense mechanisms of the respiratory tract against invading microorganisms and other inhaled particles depends on the phagocytic ability of alveolar macrophages[8,9]. These macrophages are involved in all aspects of the immune response, which include the innate and adaptive systems[9]. The failure of recognition of an invading organism resulting in failure of phagocytosis increases the susceptibility of the host to infection[10].

The aim of this study was to evaluate the correlation of macrophage phagocytosis with the mucoid phenotype of *K pneumoniae* clinical isolates and genetic characteristics,

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specifically the plasmid profile and presence of the *irp2* and $bla_{CTX-M-2}$ genes, related to the synthesis of the siderophore yersiniabactin and antimicrobial resistance, respectively, and the phenotypic feature of antimicrobial resistance.

Thirty clinical isolates of *K. pneumoniae* were obtained from different clinical samples from unrelated patients treated at three public hospitals in Recife-PE, Brazil (**Table 1**). For further identification, the cultures were biochemically analyzed using the computerized ID Mini API 32 E system (Bio Merieux, France), according to the manufacturer's instructions. The isolates had been previously characterized regarding their plasmid profile, antimicrobial resistance profile, and presence of the *irp2* and $bla_{CTX-M-2}$, as well as being analyzed using random amplification of polymorphic DNA-polymerase chain reaction (RAPD-PCR)[2,11]. All 30 isolates were analyzed concerning the mucoid phenotype of the colonies. Four of the isolates that did not display a clonal relationship[11] by RAPD-PCR were selected according to their genetic features and antimicrobial resistance to determine the rate of phagocytosis by alveolar macrophages. For determination of the mucoid phenotype, the 30 isolates were seeded onto tryptose soy agar (Oxoid, UK), and incubated at 37ºC for 18 hours.

Alveolar macrophages were obtained by bronchoalveolar lavage from rats weighing 300 g and an average age of 90 to 120 days. Anesthesia was induced intraperitoneally with chloralose (0.5%) plus uretama (12.5%), using 1 ml of anesthetic per 100 g body weight. Tracheostomy was performed and the bronchoalveolar lavage fluid was aspirated using a syringe with saline. The material obtained was centrifuged at 252 x g for 10 min and the pellet was resuspended in 1 ml of 0.2% NaCl and centrifuged for 10 min. The pellet was resuspended in 2 ml of RPMI medium (Gibco BRL, NY), pH 7.0[12].

To prepare concentrated bacterial preparations, the four selected *K. pneumoniae* isolates (**Table 2**) were used to

TABLE 1: Mucoid phenotype, source of strains, and plasmid profile of 30 clinical isolates of *K. pneumoniae* from hospitals in Recife-PE, Brazil.

Bacterial isolates	Source of strains	Mucoid phenotype	Plasmid number[(b)]	Plasmid length (kb)[(b)]
K2-R	Urine	Mucoid	4	~180, 150, 13.5, 2.3
K6-R	Respiratory secretion	Mucoid	PA[(a)]	--------
K10-R	Respiratory secretion	Non-mucoid	2	~180, 150
K11-R	Urine	Non-mucoid	4	~180, 150, 21.0, 3.4
K13-R	Respiratory secretion	Non-mucoid	PA	--------
K14-R	Respiratory secretion	Mucoid	4	~180, 150, 8.0, 3.8
K15-R	Blood	Non-mucoid	4	17.5, 13.7, 3.0, 2.8
K16-R	Urine	Non-mucoid	4	~180, 150, 21.0, 3.4
K17-R	Respiratory secretion	Mucoid	2	~180, 150, 6.5
K18-R	Urine	Mucoid	4	~180, 150, 5.0,<1.9
K19-R	Urine	Non-mucoid	2	~180, 150
K20-R	Urine	Non-mucoid	PA	--------
K21-I	Feces	Non-mucoid	3	~180, 7.0, 4.0
K22-I	Blood	Non-mucoid	1	~180
K3-C	Urine	Non-mucoid	1	~180
K4-C	Wound	Non-mucoid	3	150, 2.6, 2.3
K5-C	Urine	Mucoid	1	~180
K6-C	Urine	Mucoid	2	~180, 4.6
K7-C	Urine	Non-mucoid	1	4.0
K8-C	Urine	Mucoid	4	21.0, 4.6, 4.0, 2.6
K9-C	Urine	Mucoid	1	~180
K10-C	Urine	Non-mucoid	4	~180, 8.0, 4.0, 2.2
K11-C	Urine	Non-mucoid	2	~180, 150
K12-C	Urine	Non-mucoid	2	150, 3.9
K13-C	Wound	Non-mucoid	2	150, 4.3
K14-C	Wound	Non-mucoid	3	150, 2.9, 1.9
K15-C	Urine	Non-mucoid	2	~180, 1.9
K16-C	Urine	Non-mucoid	2	~180, 150
K17-C	Urine	Non-mucoid	2	~180, 150
K18-C	Urine	Non-mucoid	2	150, 3.9

[(a)] Plasmid absence (PA); [(b)] Souza Lopes *et al.*[11]

TABLE 2: Phagocytosis rate and phenotypic and genotypic characteristics of four clinical isolates of *K. pneumoniae* from Recife-PE, Brazil.

Bacterial isolates	Phagocytosis rate (%)	Mucoid phenotype	Antimicrobial resistance[(a)]	Plasmid number[(b)]	Plasmid length (kb)[(b)]	Gene *irp2*[(b)]	Gene $bla_{CTX\text{-}M\text{-}2(b)}$
K16-R	21.5	Non-mucoid	AMP,CO,KN,ES,CP,CTX,ATM, AMP/SUL,AMC	04	~180, 150, 21.0, 3.4	Presence	Presence
K9-C	27.43	Mucoid	AMP,CO,TT,CP, CTX,ATM	01	~180	Absence	Absence
K6-R	29.43	Mucoid	AMP	PA [(c)]	PA	Absence	Absence
K14-R	43.43	Mucoid	AMP,CO,TT	04	~180, 150, 8.0, 3.8	Absence	Absence

[(a)]CO- Chloramphenicol; **TT:** Tetracycline; **KN:** Kanamycin; **ES:** Streptomycin, **AMP:** Ampicillin; **AMP/SUL:** Ampicillin / Sulbactam; **CP:** Cefoperazone; **CTX:** Cefotaxime; **ATM:** Aztreonam; **AMC:** Amoxicillin / Clavulanate. [(b)] Souza Lopes *et al.*[11]. [(c)]PA, plasmid absence.

individually inoculate Brain Heart Infusion broth (Oxoid, UK). After incubation at 37°C for 18 hours, the bacterial cultures were centrifuged at 252 x g for 10 minutes and the supernatant was discarded. The pellet was centrifuged two more times (252 x g, 10 min) in 4 mL of phosphate buffered saline (PBS; 0.1 M, pH 7.2). Each final pellet was resuspended in 1 ml of PBS. The Neubauer chamber was used to enumerate bacteria and alveolar macrophages. Each cell suspension and 0.05% trypan blue dye was added to obtain a 1:10 dilution of the bacteria, as calculated (cells/mm^3) according to the formula n × 10 × 10 × 10^3, where n represents number of cells. The desired density was 1 × 10^6 macrophages/ml and 1 × 10^7 colony forming units (CFU)/ml for bacteria. In the experiments of macrophage phagocytosis of the *K. pneumoniae* isolates, macrophage suspensions in RPMI 1640 medium and bacterial suspensions in PBS were mixed on a glass slide for optical microscopy. Each slide was then incubated for 1 hour at 37°C in a humid chamber. Eight repetitions were done for each bacterial isolate. After an interval of one hour, the slides were stained using the Fast Panoptic kit based on hematoxylin and eosin. The slides were washed, dried at room temperature, and observed by optical microscopy to determine the rate of phagocytosis of 100 alveolar macrophages. Statistical analysis of the average rate of phagocytosis involved the analysis of variance (ANOVA).

Among the 30 *K. pneumoniae* isolates tested, nine (30%) displayed the mucoid phenotype (**Table 1 and Figure 1A**). There was no evidence of a relationship between the mucoid phenotype and the source of the bacterial isolates. Comparison of the mucoid phenotype with the plasmid profile revealed two bacterial strains (6.6%, K6 and K8-C-R) that were mucoid and lacked the 180 kb plasmid, whereas 12 isolates (40%) were non-mucoid and harbored the plasmid (**Table 1**). Additionally, among three clinical isolates without plasmids, one displayed the mucoid phenotype.

Before analyzing the phagocytic ability of alveolar macrophages, the cell viability based on the exclusion of trypan blue was determined. Viability exceeded 95% in all the samples, which indicating that the bronchoalveolar lavage fluid method was capable of recovering viable cells that were capable of phagocytosis. Alveolar macrophages activated by *K. pneumoniae* displayed rates of phagocytosis ranging from 21.5% to 43.43% (**Table 2**). **Figure 1B** displays a typical image of the phagocytosis of *K. pneumoniae* by an activated macrophage. The activated macrophage displayed cytoplasmic vacuoles that were larger and irregular in shape. To compare the average rates of phagocytosis of the four *K. pneumoniae* isolates tested, we used ANOVA. For the parameters analyzed at a significance level of 5%, the calculated F (0.56) was lower than the F table (2:57), therefore it was not necessary to perform the Tukey test since the means were not significantly different.

The phagocytosis rate of the multiresistant isolates K9-C and K16-R were 21.5% and 27.43%, respectively. These rates were lower than those found for *K. pneumoniae* isolates sensitive to antibiotics (K6-R and K14-R), suggesting a correlation between the phagocytosis rate and antimicrobial susceptibility (**Table 2**). The highest rates of phagocytosis, 27.43%, 29.43%, and 43.43% (**Table 2**) were seen in *K. pneumoniae* isolates with mucoid phenotype, whereas the lowest phagocytosis rate of 21.5% was observed in a non-mucoid strain. The findings indicated a relationship between the mucoid colony phenotype and the phagocytosis rate. The phagocytosis rate was lower in the K16-R resistant isolate that harbored the *irp2* and $bla_{CTX\text{-}M\text{-}2}$ genes. There was also no correlation between the plasmid profile and the phagocytosis rate.

Since the mucoid phenotype of colonies is an important virulence factor in *K. pneumoniae*[5], this phenotype was investigated in the 30 clinical isolates. Thirty percent of the isolates displayed the mucoid phenotype, which was consistent with the previous description of the low prevalence of mucoidy in *K. pneumoniae* clinical isolates[7]. The mucoid phenotype is determined by the *regulator of mucoid phenotype* (*rmpA*) gene found in a 1.6 kb DNA segment present in a 180 kb plasmid in *Escherichia coli* and *K. pneumoniae* strains[5,7]. The analysis of the 30 *K. pneumoniae* isolates from patients with nosocomial infections showed no correlation between the mucoid phenotype in colonies with the presence of the 180 kb plasmid, since 6.6%

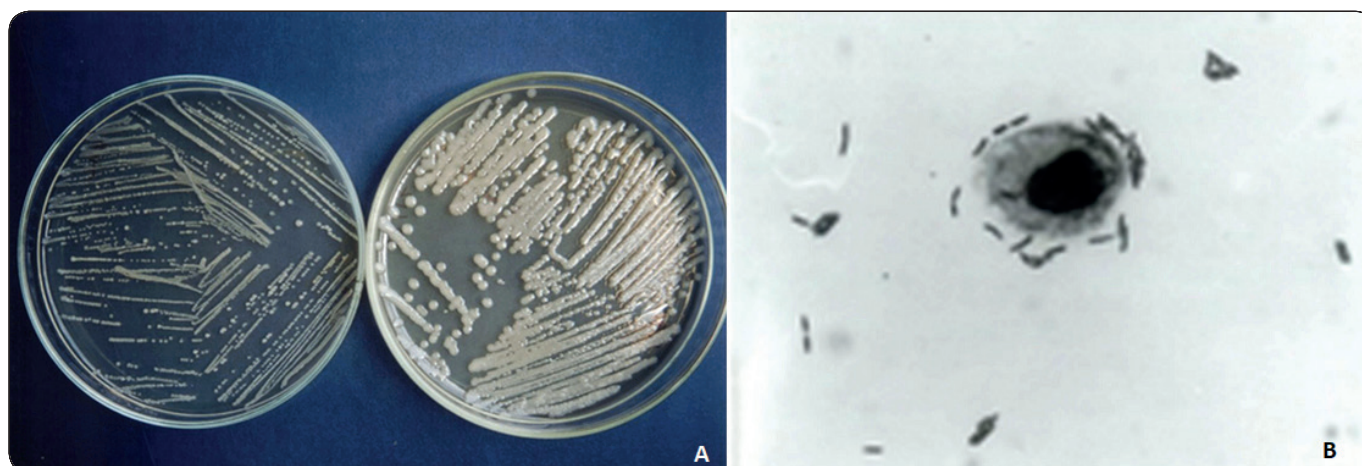

FIGURE 1: Mucoid phenotype and phagocytosis of *K. pneumoniae*. A. *K. pneumoniae* colonies presenting non-mucoid phenotype (K16-R, left) and mucoid phenotype (K-14R, right). **B.** Alveolar macrophage phagocytosing a *K. pneumoniae* K14-R bacterium.

of the mucoid isolates did not harbor the plasmid. These results suggested that the gene encoding the *K. pneumoniae* mucoid phenotype is not necessarily associated with the presence of the 180 kb plasmid, as has been described in the literature. The mucoid phenotype of the *K. pneumoniae* colonies did not decrease the level of phagocytosis by alveolar macrophages, since mucoid isolates showed the highest phagocytosis rate. However, the non-mucoid bacterial strain showed the lowest rate. Keisari *et al*.[10] demonstrated that macrophages recognize and phagocytose *K. pneumoniae* in two steps. Recognition of the capsular structures by macrophage mannose receptors is followed by opsonization by surfactant protein A (SP-A), which binds to the capsular polysaccharide of *K. pneumoniae* and the macrophage mannose receptors. According to these authors, bacterial surface specific structures may be targets for macrophage recognition by several mechanisms, as exemplified in the case of the polysaccharide capsule of *K. pneumoniae*. Therefore, in the present study, the absence of mucoid colonies of *K. pneumoniae* most likely hampered recognition by macrophages, thereby reducing the phagocytosis rate.

We observed that rat alveolar macrophages activated by *K. pneumoniae* displayed phagocytic rates ranging from 21.5% to 43.43%. This rate was lower than the 89.4 ± 6.2% rate of alveolar macrophage phagocytosis from bronchoalveolar lavage fluid of horses activated by cell wall of nonviable *Saccharomyces cerevisiae*[13]. However, the rate we observed exceeded the previously detected rates of alveolar macrophage phagocytosis *in vitro* to methicillin-resistant *Staphylococcus aureus* (12.1 ± 3.0 %) and methicillin-sensitive *Staphylococcus aureus* (10.4 ± 3.2 %)[14], and exceeds the rate of phagocytosis detected by Salgado *et al*.[15] , 6.29 ± 0.46 (MSSA) and 5.87 ± 0.34 (MRSA).

The phagocytosis rate was related to antimicrobial resistance, because the K9 and K16-R-C multiresistant strains displayed a lower phagocytosis rate than that in the antimicrobial sensitive strains. These findings may be related to the presence of the 180 kb plasmid that was present in the K9-C and K16-R isolates. This plasmid encodes several virulence factors, including mucoidy, adhesion factor, aerobactin, and ESBL TEM-5, which confers resistance to third-generation cephalosporins[5,7]. Therefore, the present results suggest that the low rate of phagocytosis of the multiresistant *K. pneumoniae* isolates was probably due to the presence of the 180 kb plasmid, which may carry genes for resistance and virulence factors, and which may consequently inhibit phagocytosis. Furthermore, K16-R, which was non-mucoid and virulent, had the lowest rate of phagocytosis by alveolar macrophages and was most resistant to antimicrobials (positive to $bla_{\text{CTX-M-2}}$ gene which encodes ESBL) and is probably the most virulent (positive to *irp2* gene which encodes the yersiniabactin siderophore)[4].

The collective data from this study indicate that several virulence factors act in parallel in *K. pneumoniae* to impair host defense. The results highlight the need for further research aimed at providing data concerning virulence factors of *K. pneumoniae*, as well as to clarify the relationship of these factors with the host defense response.

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Conflict of Interest: The authors declare that there is no conflict of interest.

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