Role of capsule in Klebsiella pneumoniae virulence: lack of correlation between in vitro and in vivo studies

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Received 6 September 2002; received in revised form 4 November 2002; accepted 5 November 2002

First published online 22 November 2002

Abstract

In vitro and in vivo models were used to investigate the role of capsule on the virulence of Klebsiella pneumoniae. We showed that capsule expression reduces dramatically the ability of the K. pneumoniae to bind to epithelial cells when compared to its non-capsulated variant. The presence/absence of capsule had no effect on the colonization of the gastrointestinal tract, while in the urinary tract we established that capsule is an important virulence factor. Our study demonstrates the caution needed when extrapolating from results of in vitro studies and emphasizes the necessity of in vivo models in studies of bacterial virulence.

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Keywords: Klebsiella; Capsule; Virulence; Adhesion; Colonization; Urinary tract infection

1. Introduction

Klebsiella pneumoniae is recognized as an important opportunistic pathogen frequently causing urinary tract infections (UTI), septicemia or pneumonia in immunocompromised individuals. It is characteristic that most K. pneumoniae infections are preceded by colonization of the patients’ gastrointestinal (GI) tract, and the GI tract of colonized patients is considered the main reservoir of transmission of the bacteria [1]. The vast majority of K. pneumoniae isolates express a pronounced polysaccharide capsule covering the entire bacterial surface, resulting in a characteristic mucoid phenotype when grown on agar plates. The capsule is generally considered to be an important virulence factor in K. pneumoniae. In vitro studies have shown that the capsule mediates protection against phagocytosis and the bactericidal effect of serum [2–5]. By using animal models the capsule has been identified as a virulence factor when K. pneumoniae was inoculated into skin lesions [4,6], lungs [7,8] and intraperitoneally [7,9,10]. The role of capsule in serum poor compartments is less clarified. Only one study has investigated the role of capsule in K. pneumoniae UTI with inconclusive results [11] and studies on the role of capsule in GI colonization are contradictory [12,13]. Interestingly, recent studies have shown that expression of capsule impedes the ability of K. pneumoniae to adhere to and invade epithelial cells in vitro [14,15]. These studies suggest that expression of capsule may be a disadvantage in serum poor host compartments by reducing the ability of the bacteria to adhere to epithelial surfaces.

The genetic and biosynthetic pathways of capsule expression in K. pneumoniae are poorly characterized. Only the K2-serotype gene cluster has been cloned and the genomic organization determined [9,16]. However, only a few open reading frames have been assigned a function and only some parts of the cluster are conserved among different capsule serotypes [14,16]. Due to the inability to construct well-defined isogenic mutants, most studies investigating the role of capsule on the virulence of K. pneumoniae are based on the use of non-capsulated variants (NCVs) arisen either spontaneously or after mutagenic treatment of the bacteria.

In the present study two K. pneumoniae strains are compared with their respective NCVs for the ability to adhere to and invade cultured epithelial cells as well as for virulence in animal models of GI colonization and UTI. Hereby we combine the use of in vitro and in vivo models to characterize the role of capsule on the virulence of K. pneumoniae.
2. Materials and methods

2.1. Bacterial strains

*K. pneumoniae* strains C105 (K35) and C3019 (K2) are streptomycin resistant human clinical isolates from stool and urine, respectively. For use in competition experiments, kanamycin resistant derivatives of C105 and C3019 were constructed by insertion of the mini-Tn5Km2 transposon into the chromosome as previously described [17]. The kanamycin resistant derivatives were found to be identical to their respective parents with respect to biochemical reactions, in vitro growth rates and virulence in mouse models of intestinal colonization and UTI (data not shown). Bacteria were grown routinely at 37°C in Luria-Bertani (LB) broth or on LB agar plates containing the appropriate antibiotics at the following concentrations: streptomycin 100 µg ml⁻¹; kanamycin 50 µg ml⁻¹.

2.2. Cell lines and culture conditions

The human ileocecal epithelial cell line HCT-8 was cultivated in RPMI 1640 medium with 25 mM HEPES supplemented with 2 mM glutamine, 1 mM pyruvate and 10% fetal calf serum. The human bladder epithelial cell line T-24 was cultivated in McCoy’s 5A medium with 25 mM HEPES supplemented with 2 mM glutamine, 1 mM non-essential amino acids and 10% fetal calf serum. The human ileocecal epithelial cell line HCT-8 was cultivated in RPMI 1640 medium with 25 mM HEPES supplemented with 2 mM glutamine, 0.1 mM non-essential amino acids and 10% fetal calf serum. The HCT-8 and T-24 cell lines were subcultivated twice a week at a ratio of 1:10 and 1:5, respectively.

2.3. Isolation of NCVs

To isolate spontaneous NCVs, the parent strains were streaked and grown overnight on Ox-heart agar plates (Statens Serum Institut (SSI)) at 37°C. The plates were subsequently incubated at room temperature for 48 h. The NCVs were obtained from non-mucoid translucent segments of colonies of the parent strain and subcultured on LB agar plates.

2.4. Visualization of capsule expression

Negative capsule staining was performed by mixing 10 µl of a bacterial suspension and 10 µl 10% nigrosin on a microscope slide. Preparations were examined by light microscopy and the presence of capsule detected as exclusion of the nigrosin dye around the bacterial cell.

The capsule swelling reaction was performed by mixing bacterial solutions with K35 or K2 capsule specific antisera (WHO, International Escherichia and Klebsiella Reference Center, SSI, Copenhagen, Denmark) on a microscope slide and the preparations examined by phase-contrast microscopy to reveal the presence of capsule by change in refractive index due to antibody binding [18].

2.5. Adhesion and invasion assays

Epithelial cells were grown to confluent monolayers in sets of 30 24-well tissue culture plates (Falcon). Approximately 2×10⁶ bacteria from an overnight culture were added to each well (approximately 30 bacteria per epithelial cell) and the plates incubated for 3 h at 37°C. The total number of bacteria at the end of the 3 h incubation period was determined by adding trypsin and Triton X-100 to a final concentration of 20% and 0.5%, respectively, to each well in the first 24-well tissue culture plate. After incubation for 10 min to separate and lyse the epithelial cells, the suspension was mixed and serial dilutions plated on selective media.

The wells in the second and third plates were washed three times with 0.5 M phosphate buffered saline (PBS) to remove unbound bacteria. To determine the number of adhering bacteria, the cells in the wells of the second plate were lysed as described above. To the wells of the third plate, fresh cell culture medium containing 100 µg of gentamicin per ml was added to kill all extracellular bacteria. After an incubation period of 1.5 h at 37°C, the wells were washed with PBS and lysed for determination of the number of intracellular bacteria. All assays were performed in triplicate and were repeated independently two times. Adhesion and invasion rates were calculated on the total number of bacteria present after the incubation period rather than to the size of inoculum. Although the inoculum size was the same, *Klebsiella* grows rapidly during the 3 h incubation period with the epithelial cells, giving a false high adhesion and invasion rate.

2.6. Mouse model of GI colonization

Six- to 8-week-old, outbred albino female mice Ssc:CF1 (SSI) were used for GI colonization experiments as described previously [19]. Briefly, mice were individually caged and given sterile water containing 5 g of streptomycin sulfate per liter throughout the experiment and fed continuously. After 24 h, 100 µl bacterial suspension containing approximately 10⁶ CFU were given per os. During the colonization experiment, cages were changed daily. At days 1, 3, 6 and 10 after inoculation, faeces was collected, homogenized in 0.9% NaCl, and serial dilutions plated on selective media.

2.7. Mouse model of ascending UTI

For UTI experiments, 6–8-week-old, outbred albino female mice Ssc:CF1 (SSI) were used. The UTI model has been described in detail previously [20]. Briefly, mice were anaesthetized by intraperitoneal administration of an 0.08 ml mixture of Hypnorm (0.315 mg fentanyl citrate ml⁻¹ and 10 mg fluanison ml⁻¹ (Janssen Animal Health)) and diazepam (5 mg ml⁻¹) in a ratio of 1:3. Anaesthetized mice were inoculated transurethrally with 50 µl bac-
A bacterial suspension containing approximately $5 \times 10^8$ CFU by use of plastic catheters. The catheter was carefully pushed horizontally through the urethral orifice until it reached the top of the bladder, and the bacterial suspension slowly injected into the bladder. The catheter was immediately removed after inoculation and the mice subjected to no further manipulations until sacrifice, 24 h after inoculation. Bladders were collected in 500 μl PBS, homogenized, and serial dilutions plated on selective media.

2.8. Statistics

The Mann–Whitney $U$ test was used for statistical evaluation. $P$ values less than 0.05 were considered statistically significant.

3. Results

3.1. Isolation and characterization of NCVs

Spontaneous NCVs of *K. pneumoniae* strains C105, C3019 and their respective kanamycin resistant derivates were obtained from non-mucoid segments of colonies of the parent strains grown on solid medium. The non-capsulated phenotype of the NCVs was confirmed by light microscopy after negative capsule staining. The parent strains were found to express a prominent capsule structure surrounding the bacterial cell, whereas a well-defined capsule structure could not be visualized in the NCVs (Fig. 1a,b). Furthermore, expression of capsular antigens in the NVCs and respective parent strains was tested by the capsular swelling technique. While the parent strains

![Fig. 1. Visualization of capsule expression in *K. pneumoniae* strain C105 (K35) and its NCV. Negative staining of capsule by nigrosin dye in C105 (a) and C105NCV (b). Capsule swelling reaction in C105 (c) and C105NCV (d) after incubation with K35 capsule antiserum. Bars represent 2.5 μm.](https://academic.oup.com/femsle/article-abstract/218/1/149/532467)

Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Median ratio NCV/Parent</th>
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<tr>
<td></td>
<td>Adhesion</td>
</tr>
<tr>
<td></td>
<td>HCT-8$^b$</td>
</tr>
<tr>
<td>C105</td>
<td>7.9</td>
</tr>
<tr>
<td>C3019</td>
<td>9.9</td>
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$^a$Representative results are from day 10 after inoculation.

$^b$HCT-8: ileocecal cells.

$^c$T-24: bladder cells.
of C105 and C3019 reacted clearly with specific K35 and K2 capsule antiserum, respectively, no reaction was observed for the NCVs (Fig. 1c,d). The NCVs were found to be identical to their respective parents with respect to biochemical reactions and in vitro growth rates. To examine the stability of the non-capsulated phenotype of the NCVs, 10 successive passages in LB broth were performed followed by subculture on solid medium. The phenotype of the NCVs was found to be stable, since reversion to the capsulated phenotype was never observed.

### 3.2. Interactions with epithelial cells

A pronounced difference in interaction with the epithelial cell lines was observed between the NCVs and their respective parent strains. The percent adhesion to the intestinal HCT-8 and bladder T-24 cell lines was approximately 10-fold higher for the NCVs compared to the parent strains (Table 1). Likewise, the percent internalization by both cell lines was significantly higher for the NCVs than for the parent strains (Table 1). To determine the role of type 1 fimbriae, (mediate adhesion to mannosylated structures), adhesion and invasions studies were also performed in the presence of 2% mannose in the cell culture medium during the incubation period. No effect of the addition of mannose to the cell culture medium on the adhesion or invasion ability of the NCVs or the parent strains was observed (data not shown).

### 3.3. GI colonization

To investigate the influence of capsule in *K. pneumoniae* GI colonization, the NCVs and parent strains were fed individually to mice and the bacterial counts of *K. pneumoniae* in faeces followed for 10 days. The NCVs were as effective colonizers as the respective parent strains and colonized the intestine at levels of about $5 \times 10^8$ CFU per gram of faeces (Fig. 2). Reversion to the capsulated phenotype was not observed for the NCVs during colonization of the intestine, as mucoid *K. pneumoniae* colonies were never detected when faeces from mice fed the NCVs were plated.

As no difference was observed in the colonization ability when fed individually to mice, the NCV and parent of each strain were also fed simultaneously to the same mice to investigate the role of capsule during competition. The NCVs and their respective wild-type strains were found to co-colonize the mice at similar levels of about $5 \times 10^8$ CFU per gram of faeces during the 10 day colonization period (Table 1). At no time significant differences in the number of wild-type and NCVs in faeces were detected, indicating that expression of capsule is not essential in *K. pneumoniae* strains C105 and C3019 during GI colonization.

### 3.4. UTI

The role of capsule in *K. pneumoniae* UTI was investigated by inoculating each strain and its respective NCV individually into sets of five mice. After 24 h, the mice were sacrificed and the bacterial counts in infected bladders determined (Fig. 3). *K. pneumoniae* was detected in the bladders of all inoculated mice. However, significantly fewer bacteria were found in bladders from mice infected with the NCVs than in mice infected with the respective parent strains (for C105, $P = 0.03$; for C3019, $P = 0.01$). The importance of capsule was furthermore established by competitive experiments. Sets of 10 mice were inocul-
ed with a mixture of equal numbers of the parent and NCV of each strain. After 24 h, the mice were sacrificed and the numbers of parent and NCV in infected bladders determined by selective plating. In all infected bladders, a higher bacterial count of the parent compared to the NCV was detected (Table 1).

4. Discussion

In the present study, the role of capsule on the virulence of two K. pneumoniae strains was investigated by use of spontaneous NCVs of the strains. The NCVs were isolated from translucent parts of bacterial colonies after prolonged growth on agar plates as described previously [3,4,7,8,15]. We confirmed the NCVs as being NCVs of the parent strains by negative capsule staining, as well as by lack of reaction with capsule specific antisera. Whether NCVs arise as a consequence of mutations in genes involved in either regulation of capsule expression or capsule biosynthesis is not known. However, the non-capsulated phenotype is stable, as neither successive passage in culture medium nor passage in mice induced reversion to the capsulated phenotype. Future genetic studies may characterize the exact nature of the NCVs.

A striking difference in the ability of the NCVs to bind to the intestinal cell line HCT-8, as well as the bladder cell line T-24, compared to their parent strains was observed in both strains. Thus, in both cell lines, the percentage of adhering bacteria was 10-fold higher for the NCVs compared to their respective parent strains. This is in agreement with recent studies reporting the expression of capsule to reduce the ability of K. pneumoniae to adhere to epithelial cell lines [14,15]. It is obvious to speculate whether the reduced ability of encapsulated bacteria to adhere may be due to masking of adhesins on the bacterial surface by the capsule. A recent study demonstrated an inverse relationship between capsule and type 1 fimbiae expression in K. pneumoniae [21]. Capsulated strains exhibited little type 1 fimbiae activity whereas type 1 fimbiae expression in non-capsulated strains was pronounced. Expression of capsule has also been found to down-regulate expression of the CF29K adhesin in K. pneumoniae, presumably at the transcriptional level [14]. The results of these studies suggest that some form of co-regulation between expression of adhesins and capsule exists in K. pneumoniae. In the present study, type 1 fimbiae did not seem to be involved in adhesion of either the NCVs or the parent strains to cultured epithelial cells, as addition of D-mannose to the cell culture medium did not influence the number of adhering bacteria.

Although traditionally considered an extracellular pathogen, recent studies have described the ability of K. pneumoniae strains to invade cultured epithelial cells [22,23]. The in vivo relevance of this phenomenon is so far uncharacterized, but the ability to invade host cells may play an important role in K. pneumoniae pathogenicity. The NCVs of both strains were found to invade both cultured epithelial cell lines in significantly higher numbers than their respective parent strains. That capsule interferes with the internalization of K. pneumoniae by cultured epithelial cells is in agreement with the results of a recent study [15].

The ability of the NCVs to colonize the intestine of streptomycin treated mice was identical to their respective parent strains. This is in accordance with a previous study where non-capsulated mutants of K. pneumoniae strains were observed to be as effective colonizers of the intestine of germfree chickens as the capsulated wild-type strains [12]. In one study, the colonization ability of a capsule defective mutant of K. pneumoniae strain LM21 was significantly attenuated [13]. Here, the capsule defective mutant was found to form aggregates during growth in the intestine, which could explain the inability of this particular mutant to effectively colonize the intestine.

To our knowledge, the role of capsule as a virulence factor in K. pneumoniae UTI has so far only been investigated in one study, and the results were inconclusive [11]. In the present study the role of capsule in UTI was examined by monitoring the infection abilities of NCVs and parent strains individually, and by competitive experiments. Mice were inoculated with a mixture of equal numbers of the NCV and parent of each strain and the ratio of NCV to parent in infected bladders subsequently monitored. This strategy is advantageous as the differences in bacterial counts in infected bladders of different mice may otherwise complicate interpretation of the results. In all infected bladders the bacterial counts of the NCVs were significantly lower than of the respective parent strain. These results establish that capsule plays a significant role in K. pneumoniae UTI.

In conclusion, the results of this study demonstrate that expression of capsule impedes interactions of K. pneumoniae with host epithelial cells in vitro. Furthermore, by use of animal models the capsule was established as a significant virulence factor in UTI, but had no influence on the ability of K. pneumoniae to colonize the GI tract. The significantly higher ability of the NCVs to adhere to and invade epithelial cells in vitro was not associated with higher infectivity in the animal models. The results of this study hereby illustrate the caution needed when extrapolating from results of in vitro studies and emphasize the use of in vivo models in studies of microbial virulence.

Acknowledgements

We thank the undergraduate student Ninell Pollas Mortensen for her participation in the work. C.S. was partially supported by Grant 1998-137-014 from the Danish Research Academy.
References


