Virulence factors (aerobactin and mucoid phenotype) in Klebsiella pneumoniae and Escherichia coli blood culture isolates

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Abstract

We examined the presence of two virulence factors in 241 blood isolates of Klebsiella pneumoniae from patients hospitalized during 1989 and 1990 in 7 French hospitals, and 125 blood isolates of Escherichia coli from one hospital. Aerobactin was scored phenotypically and genotypically with an intragenic DNA probe of 2 kb. The mucoid phenotype was assessed by culture on trypticase soy agar and by genotypic analysis (intragenic DNA probe of 235 bp). Only 6% K. pneumoniae isolates were aerobactin-positive with no significant variation according to geographical location while 20% of K. pneumoniae isolates displayed the mucoid phenotype, with a significant variation according to hospital. Aerobactin was always associated with the mucoid phenotype. The frequency of aerobactin production but not mucoid phenotype (14%) was higher among E. coli isolates (48%). They harbored two types of large plasmids. Intraperitoneal injection into mice of 10^3 cfu of K. pneumoniae producing both virulence factors demonstrated that capsular serotype K2 was the more virulent K23 and K2X.

Keywords: Klebsiella pneumoniae; Escherichia coli; Aerobactin; Mucoid phenotype; Blood cultures

1. Introduction

Klebsiella pneumoniae is one of the most common causes of Gram-negative sepsis, second only to Escherichia coli [1,2]. Klebsiella infections are associated with high morbidity and mortality [2]. The emergence of extended-spectrum β-lactamases in this species and their dissemination have greatly complicated chemotherapy and β-lactam resistant outbreaks have been reported in several countries [3]. K. pneumoniae isolates can possess multiple virulence factors. The most important seem to be the nature of the polysaccharide capsule which protects the organism against ingestion and killing by professional phagocytes [4], the lipopolysaccharide which gives protection against the bactericidal effect of serum [5-7] the adherence-mediated pili which inhibit the adhesin
process [8], the acrobatin system [9] and finally the mucoid phenotype [10]. Some of these elements are genetically determined by a large 180 kb plasmid. The presence of this plasmid enhances *K. pneumoniae* virulence in mice [9,10] but the significance of such virulence factors have not be clearly assessed in human isolates. A preliminary study showed a low frequency of nosocomial isolates producing aerobactin and with a mucoid phenotype [11]. Furthermore these phenotypes were not associated with the type of extended-spectrum β-lactamases.

In this work, we have studied the prevalence of aerobactin and mucoid phenotype among 241 *K. pneumoniae* isolates recovered from blood cultures of 241 patients hospitalized in seven French hospitals to evaluate the role of such factors in systemic disseminations and their possible linkage to antibiotic resistance (as has been suggested in *E. coli* isolates) [12,13]. Because the high frequency of these virulence factors in *E. coli* isolates (greater or equal to 30% of strains) [13,14] the frequencies of both factors were also determined from 125 *E. coli* isolates recovered from blood cultures.

2. Materials and methods

2.1. Bacterial strains and plasmids

The clinical isolates of *K. pneumoniae* from blood samples of different patients hospitalized in 7 French hospitals, between 1989 and 1990 were studied. Laboratory strains are listed in Table 1, and were kindly provided by X. Nassif (Unité de Pathogénie Microbienne, Institut Pasteur, France). All the clinical isolates (SAL89, NAT89, NANO90) were identified with API 20 E test strips (bioMérieux, Marcy l’Etoile, France). Three isolates from three hospitals were serotyped by capsular swelling tests (P. Bouvet, Unité des Entérobactéries, Institut Pasteur, France).

2.2. Antibiotic susceptibility testing

Susceptibility to antimicrobial agents was tested by the disk diffusion method on Muller-Hinton agar (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France) according to the recommendations of the Comité Français de l’Antibiogramme [15].

2.3. Phenotypic detection of aerobactin and mucoid production

The production of aerobactin was demonstrated by a cross-feeding bioassay using *E. coli* strain LG 1522 [16]. The clinical isolates to be tested for aerobactin production were grown overnight in M9 broth [17] containing the iron chelator a-a’ dipyridyl (200 μM) (Sigma Chemical Co, St Louis, MO). Strains were spotted onto hardened dipyridyl-minimal agar plates. After 18 h at 37°C satellite growth of the indicator strain LG 1522 around the disks indicated aerobactin production [9]. Each isolate was plated on trypticase soy agar (Difco, France) and the plate incubated for 18 h at 37°C as previously described [10].

<table>
<thead>
<tr>
<th>Table 1</th>
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<tbody>
<tr>
<td>Reference strains and plasmids</td>
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<tr>
<td>Strains or plasmid</td>
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<tr>
<td><strong>K. pneumoniae</strong></td>
</tr>
<tr>
<td>KP 52145</td>
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<tr>
<td>KP110</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
</tr>
<tr>
<td>HB 101</td>
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<tr>
<td></td>
</tr>
<tr>
<td>LG1522</td>
</tr>
<tr>
<td>Plasmids</td>
</tr>
<tr>
<td>pKP4</td>
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<tr>
<td>pKP 228</td>
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</table>
2.4. Genotypic detection of the aerobactin and mucoid phenotypes

**Preparation of probes**

The aerobactin probe was prepared from pKP4 DNA [9]. Plasmid DNA was digested with BglII restriction endonuclease (Bethesda Research Inc., Gaithersburg, MD) and electrophoresed on a 2% agarose gel for 2 h at 100 V/cm. The 2 kb BglII fragment was excised from the gel, electroeluted, cleaned by isopropanol precipitation, and labeled by random priming with ³²P (Amersham, Buckinghamshire, UK) [18]. The fragment was then used as a probe for the aerobactin operon. The mucoid phenotype probe was prepared from pKP228 [10], plasmid DNA was digested with BamHI and BglII restriction endonucleases, electrophoresed in a 5% polyacrylamide gel for 2 h at 250 V/cm. The probe, a 235 bp BamHI and BglII fragment, was isolated and prepared as above and was used as a probe for the mucoid phenotype operon.

**Plasmid DNA extraction and hybridization**

Plasmid DNA was extracted from *K. pneumoniae* by the rapid method of Wheatcroft and Williams [19]. Samples were subjected to electrophoresis through a 0.7% agarose gel for 2.5 h at 6.5 V/cm in TRIS acetate buffer; plasmid pIP55 was used as plasmid DNA size marker. Hybridization was performed under stringent conditions after transfer of DNA to nitrocellulose filters (Nytran membranes, Schleicher and Schuell, Inc., Dassel, Germany) by the method of Southern [20]. The probes (10⁶ dpm/ml) were hybridized to the membranes as previously described [21] under stringent conditions. The blots were heated for 2 h at 80°C prior to autoradiography. The filters were exposed to X-Omatic Kodak (Eastman Kodak Co., Rochester, NY) for 72 h at -70°C.

2.5. Experimental infection

To appreciate the role of the two virulence factors of *K. pneumoniae*, 3 clinical isolates belonging to serotype K2, K23 and K68 (strains SAL89, NAT89, NAN90, respectively) from 3 patients hospitalized in separate hospitals were selected and used for comparison. KP 52145 (aerobactin and mucoid phenotype positive) and KP 110 (aerobactin and mucoid phenotype negative) were used as controls. Cells were prepared from fresh overnight cultures in trypticase soy broth (Difco, France) at 37°C and resuspended in sterile saline. After a 5-day quarantine, OFI female mice (IFFA Credo, France) weighing 22 to 26 g each, were infected intraperitoneally with serial 10-fold dilutions of the control strains. Mortality was scored at day 15 and the 50% lethal doses (LD₅₀) were calculated by the method of Reed and Muench [22]. The LD₅₀ values obtained with the isogenic control strains were used to select two inocula (10⁵ and 10⁶ cfu per animal) for the three clinical isolates (SAL89, NAT89, NAN90). Mortality rates were scored at day 15 and were expressed comparative to the rates for the control strains at the same dilution.

3. Results

3.1. Incidence of aerobactin and mucoid phenotype

The distribution of virulence factors among 241 *K. pneumoniae* isolates from the 7 French hospitals

<table>
<thead>
<tr>
<th>Table 2</th>
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<tr>
<td>Distribution of the aerobactin and mucoid phenotypes among <em>K. pneumoniae</em> isolated from blood cultures from 7 French hospitals</td>
</tr>
<tr>
<td>Hospital No.</td>
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<tr>
<td>---</td>
</tr>
<tr>
<td>Number of isolates</td>
</tr>
<tr>
<td>Aerobactin phenotype</td>
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<tr>
<td>%</td>
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<tr>
<td>Mucoid phenotype</td>
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<td>%</td>
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*always linked to mucoid phenotype.*
is reported in Table 2. Fifteen isolates (6.2%) produced aerobactin without any significant difference according to geographical location and 49 isolates (20.3%) had a mucoid aspect on trypticase soy agar, with a significant variation according to hospital ($P < 0.01$ for hospitals no. 4 and no. 6). All isolates producing aerobactin showed a mucoid phenotype. All methods used to detect aerobactin or mucoid phenotype gave consistent results.

All 49 K. pneumoniae isolates producing aerobactin or mucoid phenotype were examined for plasmid content by gel electrophoresis (Fig. 1). All aerobactin – or mucoid – positive isolates harbored a large plasmid (s) of about 180 kb (Fig. 1). Hybridization analysis with the aerobactin and mucoid specific probes showed that these genes were harbored on these large plasmids (results not shown).

Among E. coli isolates, these virulence factors were only detected by phenotypic tests. Sixty isolates (48%) produced aerobactin and 17 (14%) were mucoid.

3.2. Antibiotic susceptibility

All 15 K. pneumoniae isolates harboring a large plasmid(s) of about 180 kb and producing both virulence factors were susceptible to multiple antibiotics (cephalotin, cefamandole, cefoperazone, cefotaxime, gentamicin, tobramycin, amikacin, trimethoprim, sulphadiazine, and pefloxacin) but somewhat resistant to amoxicillin and ticarcillin (diameters of inhibition zone sizes between 6 and 15 mm). Ten isolates were intermediate or resistant to ticarcillin (diameters of inhibition zone sizes between 7 and 15 mm), and 5 isolates were highly resistant to ticarcillin (diameters of inhibition < 6 mm). One isolate was resistant to chloramphenicol, another was resistant to kanamycin and tetracycline. Among the 49 K. pneumoniae isolates, 10 (20.4%) were highly resistant to amoxicillin and ticarcillin (diameters of inhibition zone sizes below 6 mm). Among the 125 clinical E. coli isolates, 22 (37%) were highly resistant to amoxicillin and ticarcillin (diameters of inhibition zone sizes inferior or equal to 6 mm).

### Table 3

Survival time of mice inoculated with K. pneumoniae

<table>
<thead>
<tr>
<th>No. strain</th>
<th>Capsular serotype</th>
<th>Heavy plasmid</th>
<th>Inoculum $10^4$ cfu</th>
<th>Inoculum $10^6$ cfu</th>
<th>Survival time reference strains</th>
<th>Survival time clinical strains</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>20 hours</td>
<td>4 hours</td>
<td>KP52145</td>
<td>KP110</td>
</tr>
<tr>
<td></td>
<td>K2</td>
<td>+</td>
<td>&gt; 15 days (o)</td>
<td>5 hours</td>
<td>K2</td>
<td>K2</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>&gt; 15 days (o)</td>
<td>15 hours</td>
<td>K2</td>
<td>K23</td>
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<td></td>
<td>+</td>
<td></td>
<td>&gt; 15 days (o)</td>
<td>&gt; 15 days</td>
<td>15 hours</td>
<td>15 hours</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td></td>
<td>&gt; 15 days (o)</td>
<td>6 hours</td>
<td>5 hours</td>
<td>6 hours</td>
</tr>
</tbody>
</table>

<sup>1</sup> number of mice by strain

![Fig. 1. Plasmid patterns of aerobactin production and mucoid phenotype in K. pneumoniae. Lane 1: negative control (KP 110). Lane 2: positive control (KP 52145). Lanes 3, 4, 6-8: blood culture isolates of K. pneumoniae. Lane 5: molecular size standard pIP55 (150 kb). The arrow indicates the 180 kb plasmid encoding aerobactin production and the mucoid phenotype gene.](image-url)
3.3. In vivo results

High virulence as calculated by the determination of LD₅₀ and lethality (within 40 h) was reported for K. pneumoniae strain 45145 serotype K2 which produced both two virulence factors [9,10]. We measured the survival time of mice infected intraperitoneally by two inoculum levels (10⁶ and 10⁵ cfu) of three clinical isolates of K. pneumoniae producing these two virulence factors, but belonging to three serotypes (K2, K23, and K68) (Table 3). For the high inoculum (10⁶ cfu), all mice died within between 5 and 24 h without any significant difference (χ²) according to the serotype inoculated.

All mice challenged with a low inoculum of K. pneumoniae serotype K2 died within 15 h. Mice inoculated with serotype K68 survived after 36 h. and those inoculated with serotype K23 all survived.

4. Discussion

Over the past decade Klebsiella spp. were the second most frequent cause of Gram-negative bacteriemia [2]. K. pneumoniae constitutes 82% of cases of Klebsiella bacteriemia, and interestingly early reports describe the predominance of nosocomial infections. The overall mortality was 19%. However the study described above is the largest prospective study of Klebsiella bacteriemia and does not any evidence of the involvement of the virulence factors, aerobactin and mucoid phenotype.

As for nosocomial isolates of K. pneumoniae producing an extended-spectrum β-lactamase, the proportion of K. pneumoniae isolates displaying these virulence factors was low [11]. Low frequencies of virulence factors have previously been described [24]. However other studies of aerobactin report that 15% or more of K. pneumoniae isolates are aerobactin producers [25]. The frequency did not depend of the method used and the prevalence we found for aerobactin producers among isolates of E. coli were similar to other studies which similarly described demonstrating a higher prevalence (about 50% of strains examined) [13,14,26–28].

Extended-spectrum β-lactamases are shown to be widely disseminated K. pneumoniae and frequently associated with aminoglycoside resistance [3]. In contrast, the presence of virulence factors was low, only 2% of isolates having both factors [11]. There was no correlation between the presence of virulence factors with antibiotic resistance e.g. β-lactams or aminoglycosides. Others have shown that the synthesis of virulence factors seemed to correlate to the presence of large plasmids [9,10]. A plasmid (pCCF14) encoding several virulence factors, including adhesive factor, aerobactin and an extended-spectrum β-lactamase TEM-5 [23] was shown to have a size of 185 kb and belong to Inc group FI [29]. Agarose gel electrophoresis of K. pneumoniae DNA indicated that there were various sized plasmids (Fig. 1). We have transferred the plasmids pUD18 or pUD21 encoding the extended-spectrum β-lactamase SHV-3 or SHV-4 into the highly virulent strain K. pneumoniae KP 52145 carrying the virulence plasmid. Production of aerobactin, and mucoid phenotype were lost indicating incompatibility (A. Philipp, unpublished results). The plasmids pUD18 or pUD21 belonged to Inc FI group) [29]. These preliminary results appear to indicate that are three differences amongst large plasmids mediating such virulence factors.

Interestingly the presence of such plasmid-encoded virulence factors, aerobactin and mucoid phenotype, is insufficient to give a high virulence to the producing isolate as illustrated by the assays in vivo in mice. The LD₅₀ for the virulent reference strain of serotype K2 (KP 52145) was significantly lower (LD₅₀ of 10⁴) than that of the isogenic strain without the virulence plasmid (KP 110) (LD₅₀ of 10⁹), similar to those earlier demonstrated [9,10]. Other factors, e.g. capsular antigen such as K1, K2 may thus contribute to virulence [4]. Serotype K2 has been reported to be very common in clinical strains of K. pneumoniae [25], and could be implicated as highly virulent serotype by enhancing the extracapsular polysaccharide production in relation to transcriptional regulators such as Rmp A2 [30]. Our results on virulence of three clinical isolates producing aerobactin and with a mucoid phenotype (serotype K2, K23, and K69) suggest that some extracapsular antigen are involved in virulence. Fortunately such highly virulent strains (combination of serotype K2 and plasmid-encoded virulence factors) are rare. As recently mentioned virulence in K. pneumoniae may be multifactorial [31]. Nevertheless it is necessary to
define the optimal treatment of such K. pneumoniae infections. In the largest prospective study of Klebsiella bacteremia, 14-day mortalities of patients who received monotherapy or antibiotic combination, usually β-lactam and aminoglycosides were similar [2]. However amongst a subgroup of patients who suffered from hypotension within 72 h prior to, or on the day of the positive blood culture, those who received combination therapy had significantly lower mortality than did those who received monotherapy [7]. Because the influence of the virulence factors is poorly understood, it will be necessary to explore them in an experimental model for a better treatment.

Acknowledgements

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References


