Expression of putative virulence factors by clinical isolates of *Klebsiella planticola*

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A total of 92 clinical isolates of *Klebsiella planticola* from man was examined with respect to the production of haemagglutinins and siderophores, serum resistance and distribution of capsular types. For comparison, a group of 207 clinical isolates of *K. pneumoniae* was also studied. The percentages of *K. planticola* strains able to express mannose-sensitive haemagglutination, indicating type 1 fimbriae (83%) and mannose-resistant and *Klebsiella*-like agglutination, indicating type 3 fimbriae (69%), as well as to produce the siderophores enterobactin (100%) and aerobactin (2.2%) were almost identical to those of the *K. pneumoniae* strains. Similarly, the proportion of serum-resistant strains (30%) was comparable to that of *K. pneumoniae* (25%). The capsule types most often detected in *K. planticola* were K14 (13%), K2 (9%) and K70 (9%). The incidence of K2, which is the predominant capsular type in *K. pneumoniae*, was similar in both species. These findings show that *K. planticola*, which is being detected with increasing frequency in clinical specimens from man, has the ability to express similar putative virulence factors to *K. pneumoniae*, suggesting that they may have similar pathogenicity.

Introduction

*Klebsiella* spp. are gram-negative bacteria that are frequently associated with human nosocomial infections such as urinary and respiratory tract infections and septicaemia. As opportunist pathogens, klebsiellae primarily attack immunocompromised and hospitalised patients suffering from severe underlying diseases. It is estimated that *Klebsiella* spp. cause 3–7% of all nosocomial infections [1], placing them in the top 10 of nosocomial bacterial pathogens.

*Klebsiella* infections are caused mainly by *K. pneumoniae*, medically the most important member of this genus. *K. oxytoca* has also been isolated from human infections, but to a much lesser extent. In 1981, a new species, *K. planticola*, was described [2]. Although *K. planticola* was originally thought to be restricted to aquatic, botanic and soil environments, recent studies report the isolation of this organism from human clinical specimens [3, 4]. According to these findings, *K. planticola* occurs at a surprisingly high frequency of 3.5–18.5% among clinical isolates of *Klebsiella* spp. It is possible that, in addition to *K. pneumoniae* and *K. oxytoca*, *K. planticola* is able to cause human infections.

However, as most of the isolates have been obtained from polymicrobial specimens, the pathogenic significance of *K. planticola* in human infections is difficult to estimate. Moreover, nothing is known yet of the ability of *K. planticola* strains to express factors thought to contribute to the pathogenicity of *Klebsiella* spp. [5]. Among these virulence factors, the production of capsular polysaccharides has been regarded as the main determinant of *klebsiella* pathogenicity; 77 different capsular types have been reported [6], certain serotypes appearing to be more virulent than others [7, 8].

A number of other virulence factors have been described. Most strains of *K. pneumoniae* produce two different types of fimbriae that mediate adhesion to host cells. Type 1 (or common) fimbriae cause mannose-sensitive haemagglutination (MSHA) and play an important role in urinary tract infections [9–11]. Type 3 fimbriae mediate mannose-resistant and *Klebsiella*-like agglutination of tanned erythrocytes (MR/K-HA) and are reported to correlate with catheter-associated bacteriuria caused by *Providencia stuartii* [12]. Another characteristic regarded as a *klebsiella* virulence factor is the ability to resist the bactericidal effect of human serum. In *K. pneumoniae*, serum resistance properties are more common among isolates from clinical specimens than in faecal or environmental isolates [13]. Furthermore, *Klebsiella* isolates have been shown to produce high-affinity iron-chelating siderophores [11, 14, 15]. While
Materials and methods

Bacterial strains

A total of 92 K. planticola isolates from human clinical specimens was investigated; most of them (85 isolates) have been described previously [3]. All isolates were identified by the API20E system (API bioMérieux, Germany) with additional tests (fermentation of melezitose and L-sorbose, gas production from lactose) described previously [3]. All isolates were checked with K. planticola specimens was investigated; most of them (85 isolates)

The comparison group consisted of 207 isolates of K. pneumoniae from human urinary tract and wound infections, bacteraemia and pneumonia.

All isolates were stored in brain heart infusion broth containing glycerol 30% at −80°C until required. Escherichia coli strains H1939, H1887, H1886 and K311 were kindly provided by K. Hantke, University of Tübingen, Germany. E. coli F205 was a gift from P. Williams, University of Leicester.

Capsule typing

The isolates were serotyped by the capsule swelling method as described by Ullmann [17]. Polyvalent antcapsular sera were used for screening and mono-specific sera for typing.

Haemagglutination assay

The expression of MSHA indicating type 1 fimbriae and M R/K-HA indicating type 3 fimbriae was examined as described previously [18]. MSHA was assessed on guinea-pig erythrocytes and M R/K-HA was determined on tanned ox erythrocytes. Bacteria were grown statically for 48 h, then 50 μl of bacterial suspensions (c. 10¹¹ bacteria/ml) and 50 μl of erythrocytes (5 × 10⁹/ml) were mixed on porcelain tiles with rocking and observed for 3 min at room temperature. A agglutination was finally read after further incubation for 10 min at 4°C.

Determination of siderophore production

Enterobactin and aerobactin production was detected by the cross-feeding bioassay of Hantke [19] as described elsewhere [20]. Nutrient agar supplemented with 2,2′-dipyridyl (final concentration 200 μM) served as iron-restricted agar medium. E. coli H1887 (ColV−, Aer−, Iut−, FepA−, Fiu−, Cir−, aroB) was used as the indicator strain for aerobactin production and strain H1939 (FepA+, Fiu−, Cir−, FhuA−, FhuB−, aroB) for enterobactin. Aerobactin production was counter-checked with E. coli strain H1886, which is the Iut− parent strain of H1887. Strain K311 (pColV-K311) served as a positive control in the aerobactin test. Each isolate was tested twice.

Ferric aerobactin receptor

The method of Carbonetti and Williams [21] was used to detect the aerobactin receptor. Because the receptor for the ferric-aerobactin complex is also the receptor for cloacin DF13, the isolates were tested for their sensitivity to this bacteriocin. Briefly, the strains to be tested were grown in nutrient broth (Difco) which had been rendered iron-deficient by the addition of 2,2′-dipyridyl (final concentration 200 μM) to induce siderophore receptor synthesis. After overnight growth, the bacteria were spread as lawns on to nutrient agar. A crude preparation of cloacin DF13 (20 μl) was then spotted on to each lawn. After incubation, strains expressing the ferric aerobactin receptor were indicated by a clear zone in the lawn of growth.

Cloacin was prepared by adding mitomycin C (0.2 μg/ml) to a growing culture in nutrient broth of the producer strain E. coli F205 (CloDF13::Tn901). After incubation overnight with shaking at 37°C, cells were removed by centrifugation and the cloacin-containing supernate was sterilised by filtration.

Serum bactericidal assay

The susceptibility of bacteria to human serum was determined by the method of Hughes et al. [22] with slight modifications [13]. Bacteria were diluted to 2 × 10⁶ cells/ml in physiological saline. Then 25 μl of bacterial suspensions and 75 μl of normal human serum (NHS) were placed in the wells of microtitration trays, mixed and incubated at 37°C. Samples were taken immediately after mixing and after incubation for 1, 2 and 3 h, and serial dilutions were plated on brain heart infusion agar for colony counts. Responses were graded from 1 to 6 [22] as follows (each grade is shown by an example strain in Fig. 1a): grade 1, viable counts (VC) after 1 and 2 h were <10% of the inoculum, after 3 h <10%; grade 2, VC after 1 h were 10–100%, after 3 h <10%; grade 3, VC after 1 h were >100%, after 2 and 3 h <100%; grade 4, VC after 1 and 2 h were >100%, after 3 h <100%; grade 5, VC after 1, 2 and 3 h were >100%, but VC fell at some time during the 3-h period; grade 6, VC after 1, 2 and 3 h were >100% of the inoculum and rose throughout the 3-h period. Each strain was tested three times.

the role of the catechol-type siderophore, enterobactin, in virulence is still uncertain, the contribution of the hydroxamate-type siderophore, aerobactin, to virulence has been demonstrated clearly [16].

The present study was undertaken to evaluate whether K. planticola expresses any of the aforementioned klebsiella virulence factors. Clinical isolates of K. planticola were compared in this respect with a group of clinical isolates of K. pneumoniae.
Statistical analysis

The significance of differences between groups of bacteria was evaluated by Yates’ corrected $\chi^2$ for $2 \times 2$ contingency tables.

Results

A total of 92 clinical isolates of K. planticola was investigated with respect to the expression of putative klebsiella virulence factors. The incidence of isolates expressing such markers was compared with that observed among 207 K. pneumoniae isolates from human infections.

Capsule types

The capsule swelling method was able to type the K. planticola isolates (96%) as readily as it did K. pneumoniae. The distribution of the capsular types is shown in Table 1; 32 different K types were observed among the K. planticola isolates investigated. In this species, serotype K14 was the commonest (13%), followed by K2 and K70 (8.7% each). K2 was also the commonest K type in K. pneumoniae (13.5%), but serotype K70 was not detected in any isolate of this species. The seven most prevalent K types accounted for >50% of the K. planticola isolates investigated, whereas the serotype distribution among K. pneumoniae showed greater homogeneity.

Fig. 1. Susceptibility of clinical K. planticola (◼) and K. pneumoniae (◼) isolates to normal human serum. (a) Grades of responses; (b) distribution of responses among isolates.
Table 1. Distribution of capsular types among clinical isolates of K. planticola and K. pneumoniae

<table>
<thead>
<tr>
<th>Capsule type</th>
<th>K. planticola (n = 92)</th>
<th>K. pneumoniae (n = 207)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K14</td>
<td>12 (13.0)</td>
<td>9 (4.3)</td>
</tr>
<tr>
<td>K2</td>
<td>8 (8.7)</td>
<td>28 (13.5)</td>
</tr>
<tr>
<td>K70</td>
<td>8 (8.7)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>K33</td>
<td>6 (6.5)</td>
<td>3 (1.4)</td>
</tr>
<tr>
<td>K7</td>
<td>5 (5.4)</td>
<td>2 (1.0)</td>
</tr>
<tr>
<td>K65</td>
<td>5 (5.4)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>K5</td>
<td>4 (4.3)</td>
<td>4 (1.9)</td>
</tr>
<tr>
<td>K13</td>
<td>4 (4.3)</td>
<td>2 (1.0)</td>
</tr>
<tr>
<td>K32</td>
<td>4 (4.3)</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td>K52</td>
<td>1 (1.1)</td>
<td>3 (1.4)</td>
</tr>
<tr>
<td>K27</td>
<td>1 (1.1)</td>
<td>4 (1.9)</td>
</tr>
<tr>
<td>K54</td>
<td>1 (1.1)</td>
<td>7 (3.4)</td>
</tr>
<tr>
<td>K1</td>
<td>1 (1.1)</td>
<td>6 (2.9)</td>
</tr>
<tr>
<td>K12, K16</td>
<td>1 of each (1.1)</td>
<td>5 (2.4)</td>
</tr>
<tr>
<td>K27</td>
<td>1 (1.1)</td>
<td>4 (1.9)</td>
</tr>
<tr>
<td>K52</td>
<td>1 (1.1)</td>
<td>3 (1.4)</td>
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<tr>
<td>K21, K45, K55, K67</td>
<td>1 of each (1.1)</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td>K11, K34, K50, K68, K72</td>
<td>1 of each (1.1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Untypable</td>
<td>4 (4.3)</td>
<td>16 (7.7)</td>
</tr>
</tbody>
</table>

Siderophore production

Except for two K. pneumoniae isolates, all K. planticola and K. pneumoniae isolates investigated produced enterobactin (enterochelin) under iron-restricted conditions (Table 1). In both species, synthesis of aerobactin was rarely detected - 2% of the K. planticola isolates and 5% of the K. pneumoniae isolates were aerobactin-positive, the difference was not statistically significant (p > 0.25). Although both the aerobactin-positive K. planticola isolates expressed the aerobactin receptor, this was true of only nine of the 11 aerobactin-producing K. pneumoniae isolates (data not shown).

Discussion

Although originally thought to be an exclusively environmental species without clinical significance, there is growing evidence that K. planticola should be regarded as an opportunist pathogen in hospitals [3, 4, 23, 24]. Its incidence among clinical isolates of Klebsiella spp. ranges from 3.5% to 19% [3, 4, 24]. The reasons for this emergence are unknown. It is very probable that the rate of K. planticola isolations has increased because more investigators, being unable to identify this Klebsiella sp. by conventional laboratory identification tests, are supplementing them with additional differentiating tests.

However, it is not known whether K. planticola expresses features thought to represent klebsiella virulence factors [5]. The incidences of particular capsule types, fimbriae, serum resistance properties and siderophore synthesis have been examined in K. pneumoniae and K. oxytoca, but not in K. planticola. If K. planticola is a human pathogen like K. pneumoniae, it should express klebsiella virulence factors at incidences similar to those of K. pneumoniae; therefore, the present study compared clinical isolates of both species in this regard.

In K. pneumoniae, the most frequent capsular serotype by far was K2 (13.5%). This is in agreement with other studies, which show this K antigen to be very common among clinical Klebsiella isolates [25–27]. In K. planticola by contrast, K14 was the predominant capsular type (13%). However, the frequency of K. planticola isolates expressing the K2 antigen (8.7%) was similar to that in K. pneumoniae. This sheds some

Haemagglutination

MSHA was detected in 83% of the K. planticola isolates investigated (Table 2). MR/K-HA was observed in 69% of these isolates. The incidences of MSHA and MR/K-HA in K. planticola were very similar to those found in the clinical K. pneumoniae isolates (86% and 70%, respectively). Similarly, the proportion of isolates showing neither MSHA nor MR/K-HA was identical in both species (each 4.3%).

Serum resistance properties

The susceptibility of the isolates to the bactericidal action of NHS was determined over a period of 3 h. The responses were arranged into six grades as shown in Fig. 1a. An isolate was classified as being highly resistant (grade 5 or 6). The distribution of responses to pooled NHS is shown in Fig. 1b. Apart from a higher incidence of grade 3 susceptibility in K. planticola, both species exhibited a similar distribution of response grades. Serum resistance (grades 5 and 6) was observed among 25% of the K. pneumoniae and among 30% of the K. planticola isolates (Table 1); the difference was not statistically significant (p > 0.25).
light on the pathogenic capability of K. planticola, as serotype K2 and K1 strains are known to be much more virulent than other capsular types [7].

Among the three most common K. planticola serotypes, K 70 was found as often as K2 (8.7%). Capsular type K70 appears to be closely associated with this species, as this serotype was not detected among the 207 K. pneumoniae isolates investigated.

With respect to other putative klebsiella virulence factors, clinical isolates of K. planticola were very similar to clinical strains of K. pneumoniae. The incidences of MSHA and MR/K-HA, as well as of enterobactin-production, were almost identical for both species. Serum-resistant isolates and isolates synthesising aerobactin were almost as common in K. planticola as in K. pneumoniae.

Taken together these findings suggest that the virulence capabilities of K. planticola are similar to those of K. pneumoniae. Furthermore, the data suggest that K. planticola should be regarded as a pathogenic Klebsiella sp. While only a few of the isolates described to date were from monomicrobial specimens, and thus could be clearly identified as the causative agents of infections [3], the number of reports describing the isolation of K. planticola from human clinical specimens is growing steadily. It is likely that the frequency at which K. planticola is isolated from clinical infections will increase as the number of bacteriological laboratories able to perform the appropriate cultural tests grows.

We thank Andrea Hölzgen for her expert technical assistance.

References