Molecular Typing of Hospital Acquired Klebsiella Isolates from Mansoura University Children Hospital (MUCH)

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ABSTRACT

Nosocomial isolates of Klebsiella species collected from Mansoura University Children Hospital during the period of the study were analysed in order to determine their epidemiological relatedness and genetic characteristics. Klebsiella isolates were characterized by biotyping, antibiotyping, plasmid profile and multiplex PCR. Klebsiella pneumoniae was the most frequent isolated nosocomial pathogen (17.0% of the total isolates), while Klebsiella oxytoca represented only 1.57%. The majority of the clinical isolates were resistant to multiple antibiotics, in particular to \(\beta\)-lactams, and 51.3% were found to be extended-spectrum \(\beta\)-lactamase (ESBL) producers. ESBL-phenotype by double disk synergy (DDS) test was associated with multiplex PCR (mPCR) pattern B; (contain \(wzc\) and \(bla_{CTX-M}\) genes), with the exception of two isolates which were ESBL-positive by DDS, and found belonging to mPCR pattern A; (contain \(wzc\) gene only). Plasmid analysis was efficient to differentiate \(K.\) pneumoniae strains; it delineated 8 distinct plasmid profiles. However, mPCR appeared less discriminatory, since it stated only 3 genotypic groups. We also investigated an outbreak caused by a multiresistant \(K.\) pneumoniae involved 7 patients admitted to the pediatric ICU of MUCH. We assumed that the outbreak was related to a healthcare worker with onychomycosis colonized in her hands with the same ESBL-producing Klebsiella pneumoniae (ESBL-Kp) strain. Molecular typing of the isolates revealed clonal similarity between the recovered strain and the isolates of the 7 cases. Plasmid analysis and mPCR proved to be effective techniques in discriminating unrelated Klebsiella pneumoniae isolates. We concluded that \(K.\) pneumoniae infections in MUCH have been caused by a variety of strain genotypes. So, tracing of the sources and revising our infection control policies were strictly implemented.

Keywords: Klebsiella, Klebsiella pneumoniae typing, \(\beta\)-lactamase, ESBL, nosocomial infections, molecular typing.

INTRODUCTION

Klebsiella species are ubiquitous in nature. Their habitat includes both the external environment, where they can be found in soil, on plants and in surface waters \(^1\) and also the mucosal surfaces of mammals, including the mucosal surfaces of gut, nasopharynx, etc. \(^2\). Infections caused by \(Klebsiella\) pneumoniae (\(K.\) pneumoniae) involve predominantly the respiratory and urinary tract, surgical wounds systemic infections, bacteraemia and sepsis.\(^3,4\)

It is estimated that Klebsiella species cause 3-7% of all nosocomial infections, placing them in the top 10 of nosocomial bacterial pathogens.\(^8\). Klebsiella spp. are responsible for infections primarily in intensive care units and pose a major risk especially to the immunocompromised patients treated in neonatal ICUs (NICUs)\(^6\). During three last decades etiology of nosocomial infections significantly changes due to employment of new wide-spectrum antibacterial preparations and also appearance of multi-resistant strains in environment and the increase in number of invasion procedures.\(^7\).

First described in Germany (1983) and France (1985) among Klebsiella spp., ESBLs exist in every region of the world and in most genera of enterobacteria.\(^8\). After the use of extended-spectrum cephalosporins, ESBL-Kp has become an increasingly serious problem worldwide.\(^8,10\). From an epidemiological point of view, it is often necessary to determine the clonality of the strains. This is particularly important in endemic and epidemic nosocomial outbreaks of Klebsiella infections to improve the management of such outbreaks.\(^8\). Methodologies for typing have greatly improved the ability to perform epidemiologic investigations of nosocomial infections, particularly in the neonatal and pediatric ICUs. Molecular typing should be used in conjunction with clinical and epidemiologic data and should supplement, and not be a substitute for, a
carefully conducted epidemiologic investigation\[11\]. The aim of this work was to isolate Klebsiella species from MUCH with detection of different factors increasing the risk of their acquisition. Also, detection of possible hospital outbreaks caused by any Klebsiella species. Moreover, tracing the source of infection within health care workers and the environment, and typing of the isolated strains by different typing methods as biotyping, antibiotic sensitivity pattern, plasmid analysis and multiplex PCR.

MATERIALS AND METHODS

2.1. Patients: This study was conducted from July, 2008 till August, 2009 in Mansoura University Children Hospital. Samples were collected from patients admitted at different wards of MUCH and fulfilling the criteria of nosocomial infection, as a routine work according to the policies of Microbiology Diagnostics and Infection Control Unit (MDICU), Medical Microbiology and Immunology department, Mansoura University.

2.2. Samples and processing: The samples included blood, urine, wound and throat swabs, parts of urinary and vascular catheters, sputum, endotracheal aspirates (ETA) and CSF. All collected samples were processed in MDICU laboratory. Identification of Klebsiella species was done by colony morphology, Gram-stained films and API20E (Bio-Mérieux). Antibiotics sensitivity testing was done by the disk diffusion (Kirby-Bauer) method. The antibiotic disks were selected according to the protocol of MDICU, as recommended by the Clinical and Laboratory Standards Institute \[12\].

2.3. Phenotypic detection of ESBL production: Double-disk synergy (DDS) method for detecting amoxicillin/K+ clavulanate synergy was used \[13\].

2.4. Molecular methods: 2.4.1. Plasmid analysis \[14\].
2.4.2. Multiplex PCR; DNA extraction was performed by QIAamp DNA kits® with the use of the following primer pairs: 5'-GAT ACA GGT GTA TTG TCG C-3' and 5'-GAG CTC TAT ATG TTG GAT GC-3' for wzc locus (K1 gene of capsule biosynthesis)\[15\], 5'-TTG AAT TCT CGT CTC TTC CAG A-3' and 5'-TTG GAT CCC AGC GCT TTT GCC GTC TAA G-3' for blac\[CTX-M\] locus (ESBL CTX-M group 1) \[16\] and 5'-CTA CCG CAG CAG AGT CTT TG-3' and 5'-AAC CAG TTT TGC CTT ACC AT-3' for blai\[IMP\] locus (Carbapenem resistance)\[17\]. Nucleic acid amplification was performed by QIAGEN Multiplex PCR kit®. According to the manufacture's instructions, thermal cycler program was adjusted as: denaturation at 95°C for 15 minutes, three step cycling x45 times: (denaturation at 94°C for 30 seconds, annealing at 60°C for 90 seconds, extension at 72°C for 90 seconds and Final extension at 72°C for 10 minutes).

2.5. Typing of isolates: 2.5.1. Biotyping: API 20E identification system.
2.5.2. Antibiotic sensitivity testing (resistotyping).
2.5.3. Molecular typing methods were carried out by:
2.5.3.1. Plasmid analysis.
2.5.3.2. Multiplex PCR.

RESULTS Eighty three Klebsiella species were recovered among 445 nosocomial pathogens. So, the relative nosocomial infection rate of Klebsiella was 18.6% (83/445) and this was considered the most common nosocomial pathogen in MUCH during the study period. Out of the 83 Klebsiella isolates, 76 strains were K. pneumoniae (91.5%) and 7 strains were identified as Klebsiella oxytoca (8.4%). K. pneumoniae was the most frequently isolated nosocomial species in MUCH, it represented 17.0% of the total nosocomial isolates, whereas, Serratia marcescens, K. oxytoca, Proteus mirabilis and Proteus vulgaris were the least frequently isolated, they represented 0.67%, 1.57%, 2.69% and 3.14%, respectively.
Table (1): Frequency of Klebsiellae isolation according to infection sites:

<table>
<thead>
<tr>
<th>Infection site</th>
<th>Total nosocomial isolates</th>
<th>Klebsiellae positive cultures</th>
<th>Other pathogens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Klebsiellae</td>
<td>K. pneumoniae</td>
<td>K. oxytoca</td>
</tr>
<tr>
<td>SSI</td>
<td>40</td>
<td>9</td>
<td>22.5</td>
</tr>
<tr>
<td>BSI</td>
<td>103</td>
<td>15</td>
<td>14.5</td>
</tr>
<tr>
<td>URTI</td>
<td>65</td>
<td>5</td>
<td>7.69</td>
</tr>
<tr>
<td>LRTI</td>
<td>93</td>
<td>23</td>
<td>24.7</td>
</tr>
<tr>
<td>GIT</td>
<td>42</td>
<td>9</td>
<td>58.8</td>
</tr>
<tr>
<td>CNS</td>
<td>11</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>UTI</td>
<td>91</td>
<td>22</td>
<td>24.1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>445</td>
<td>83</td>
<td>18.6</td>
</tr>
</tbody>
</table>

This table shows *K. pneumoniae* represented 20.0%, 12.6%, 7.69%, 23.6%, 21.4% and 20.8% of nosocomial surgical site infections (SSI), blood stream infections (BSI), upper respiratory tract infections (URTI), lower respiratory tract infections (LRTI), gastrointestinal tract (GIT) infections and urinary tract infections (UTI), respectively, while *K. oxytoca* represented 2.5%, 1.94%, 0%, 2.5%, 0% and 3.29% of nosocomial SSI, BSI, URTI, LRTI and UTI, respectively. Klebsiella species didn’t cause any CNS infections in MUCH during the period of the study.

We selected 50 *K. pneumoniae* isolates which possessed resistance patterns regardless the source or the sample, and excluded the remainder multi-sensitive 33 strains. We classified the selected 50 isolates randomly into 11 resistance patterns (resistotypes), termed from R1 to R11 (table 2).

Table (2): Antibiotic sensitivity patterns (Resistotypes) of the isolated *K. pneumoniae*:

<table>
<thead>
<tr>
<th>Pattern (Resistotypes)</th>
<th>Antibiotyping Profiles</th>
<th>No. of Isolates</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>S R R R S S S S S S</td>
<td>11</td>
<td>22</td>
</tr>
<tr>
<td>R2</td>
<td>S R R R R R R R R R</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>R3</td>
<td>R R R R S S R S S S</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>R4</td>
<td>S R R S S S S S S R</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>R5</td>
<td>R R R R R R R R S S</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>R6</td>
<td>S R R R S R S S R S</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>R7</td>
<td>S R R R R R R R S S</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>R8</td>
<td>S S R S R S R S S S</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>R9</td>
<td>R R R S S R S S S S</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>R10</td>
<td>S S R S R R S S S S</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>R11</td>
<td>R R R R R R R R R R</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>

The table shows that *K. pneumoniae* with resistotype R1 was the most prevalent type (22%), then R2 and R4 (14% each) and R6 (12%). The least type was R9 (4%).

**Description of the outbreak:**
During the period of the study, and in January (2009), we noticed increased the prevalence of ESBL-Kp in the PICU, involved 7 infections (4 blood stream infections, 2 UTIs and one case of pneumonia). The first case occurred in 12 January, 2009 and concerned a 2-year female child admitted to PICU due to diabetic keto acidosis (DKA). Six days later, ESBL-Kp strain was isolated from her blood sample. The patient had no history of previous hospitalization in another hospital. Later, the strain progressed to involve another 6 patients in the same ICU. The infection was thought to be a contributory factor in the death of two of the patients with blood stream infection and the patient with pneumonia.

**Biotyping:**
Three different biotypes were distributed among the 50 *K. pneumoniae* isolates on the basis of the formulated profile found after positive and negative reactions in the API20E identification system. These biotypes were arbitrarily designated B1, B2 and B3. Forty three (86%) of *K. pneumoniae* isolates in MUCH belonged to biotype B1, four (8%) isolates belonged to biotype B2 and three (6%) isolates belonged to biotype B3.

**Plasmid profile analysis:**
The results obtained by agarose gel electrophoresis of plasmid isolated from 50 strains of *K. pneumoniae* are demonstrated in table (3) and figure (1).

Plasmid profiles demonstrated that 47 (94%) out of tested 50 isolates contained plasmids and 3 (6%) isolates lacked any plasmids. Depending upon sizes and the number of plasmids, the 47 plasmid positive isolates possessed from 1 to 5 plasmids of different sizes, producing 7 plasmid profiles (designated P1 to P7) and profile P8 lacked any plasmid.

<table>
<thead>
<tr>
<th>Plasmid profile</th>
<th>Plasmid size(s) (base pair)</th>
<th>No. of isolates</th>
<th>Resistance profile</th>
<th>ESBL-positive</th>
<th>ESBL-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>550, 2000</td>
<td>10</td>
<td>R1</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>P2</td>
<td>550, 1300, 2000, 2800</td>
<td>9</td>
<td>R2</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>R4</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>P3</td>
<td>2000, 1300</td>
<td>7</td>
<td>R6</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>P4</td>
<td>550, 1650, 3300</td>
<td>5</td>
<td>R5</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>P5</td>
<td>550, 1300, 1650, 2800</td>
<td>3</td>
<td>R11</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>P6</td>
<td>3300</td>
<td>3</td>
<td>R9</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>R8</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>P7</td>
<td>550, 1300, 2000</td>
<td>4</td>
<td>R7</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>P8</td>
<td>No plasmids</td>
<td>2</td>
<td>R3</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>R1</td>
<td>0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Figure 1: Agarose gel electrophoresis of plasmid.**
Typing by multiplex PCR:
The results obtained by agarose gel electrophoresis of chromosomal DNA amplified by mPCR from 50 strains of *K. pneumoniae* demonstrated in table (4) and figure (2).

Table (4): Multiplex PCR typing and resistance profiles of *K. pneumoniae* isolates:

<table>
<thead>
<tr>
<th>mPCR profile</th>
<th>Amplicon sizes (bp)</th>
<th>Total No.</th>
<th>Corresponding Resistotype</th>
<th>ESBL by DDS</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>350</td>
<td>20</td>
<td>R1</td>
<td>-</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R3</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R8</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R9</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R5</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R7</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>350 and 587</td>
<td>27</td>
<td>R2</td>
<td>+</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R4</td>
<td>+</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R5</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R6</td>
<td>+</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R7</td>
<td>+</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R9</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>C</td>
<td>350, 415 and 587</td>
<td>3</td>
<td>R11</td>
<td>+</td>
<td>3</td>
</tr>
</tbody>
</table>

Figure 2: Agarose gel electrophoresis of amplified DNA.
Lane 1 shows x 164 HaeIII DNA molecular marker, Lanes 2, 4, 7 and 11 belong to mPCR pattern A, Lanes 8 and 10 belong to mPCR pattern B. Lanes 3, 5 and 6 belong to mPCR pattern C.

DISCUSSION

In pediatric wards, nosocomial Klebsiella infections are especially troublesome particularly in premature infants and ICUs.[18] Nosocomial outbreaks due to ESBL-producing members of the family Enterobacteriaceae have been mostly described in adult, pediatric or neonatal ICUs and nursing homes.[19]

In this study, *K. pneumoniae* was the most frequent isolated pathogen in MUCH accounting for 17.0% of the total nosocomial isolates. This finding is consistent with findings of other previous studies[20,21]. Such data are not going with the results of other some studies[22,23]. This difference may be attributed to that all the studied groups in such two studies were admitted to ICUs with exposure to more invasive procedures.

In our work, the most effective tested drug against *K. pneumoniae* infection was imipenem (96.1%), followed by tazobactam (86.8%) and the least effective was ampicillin (3.9%). These results prove the results of Martins-Loureiro et al.[24] & Garcia-Rodriguez and Jones.[25], who reported that resistance rate to imipenem, were 9.7 and 2% respectively. But in contrary, others reported that all their isolates were sensitive to
imipenem\textsuperscript{[26,27,28]}, although, the risk factors related to carriage state in such studies were evident including being bedridden, prolonged hospital stay, exposure to multiple antibiotics and protracted contact with healthcare personnel.

The ESBL production among \textit{K. pneumoniae} in our work was reported in 51.3\%. This is concomitant with the results of Lincopan \textit{et al.}\textsuperscript{[27]}, who found that the incidence of ESBL among \textit{K. pneumoniae} was 45.4\%. On the other hand, this is higher than other results\textsuperscript{[21,22,29]}. Sorry to say, higher incidences were detected in many investigations\textsuperscript{[30,31,32,33,34]}.

We reviewed medical records concerning antibiotics use; 88.2\% of infected patients were under antibiotic coverage. The 3\textsuperscript{rd} generation cephalosporins were the most used antibiotics (35.5\%) among these patients. Moreover, they were also the mainly used antibiotics in patients harbored ESBL-Kp strains (48.7\%). Compared with a survey performed by Pai \textit{et al.}\textsuperscript{[35]}, the prevalence of ESBL in \textit{K. pneumoniae} in our study was much higher (8.7\%, vs 51.3\% respectively).

**Epidemiological investigation and control program during outbreak:**

During January (2009), we detected an outbreak of ESBL-Kp in the PICU, involving 7 patients. The pediatric staff was notified of the outbreak. The MDICU infection control team of MUCH was alerted, and the infection control practitioner conducted multiple visits to the PICU. Active surveillance of colonized patients was carried out after the first case was detected; rectal swabs were performed to all PICU patients at admission and weekly thereafter. Contact precautions were implemented with all patients. In addition to clinical samples, pharyngeal and rectal screening samples were obtained prospectively twice a week from all patients admitted to the PICU or to the pediatric ward, where the children were transferred after a stay in the PICU.

Hand carriage by staff is presumed to make a large contribution to cross-contamination\textsuperscript{[36]}. We tried to identify members of medical and nursing staff, and radiologists who were significantly involved with cases. They were investigated by hand swabbing.

We assumed that the outbreak was related to a healthcare worker female with onychomycosis colonized with ESBL-producing \textit{K. pneumoniae}, since she was in close contact with all patients in the PICU. The recovered strain, by different typing methods, shared the same biotype (B1), resistotype (R2), plasmid profile (P2) and mPCR DNA pattern (pattern B), with the detected etiological ESBL-Kp strain. Moreover, the outbreak was controlled after she was removed from direct patient contact, during which, she received oral ciprofloxacin, topical tobramycin (after doing antibiotic and sensitivity testing), and oral fluconazole for the underlying onychomycosis.

In this study, we performed a head-to-head comparison of different epidemiological typing methods of the selected 50 \textit{K. pneumoniae} isolates.

In the present study, 94\% of \textit{K. pneumoniae} isolates harbored small molecular weight plasmids, and 46\% of these isolates were ESBL-positive. The presence of high molecular weight plasmids in clinical isolates of \textit{K. pneumoniae} has been observed in several countries and they were frequently associated with the production of ESBL\textsuperscript{[37,38]} The method we followed for plasmid analysis is suitable only for small scale plasmids, so, we didn’t detect large-sized plasmids.

In this work, the ESBL-phenotype was not observed in plasmid-free cells. This may be related to either the small number of plasmid-free isolates in this study (only three isolates), or the fact that ESBL-encoding resistance is present in plasmids.

We used mPCR for detection of genes in \textit{K. pneumoniae}. Specific primers fit to detect these genes have been used; \textit{wzc} (K1 gene of capsule biosynthesis, a housekeeping gene for Klebsiella), bla\textsubscript{CTX-M} (ESBL CTX-M group 1) and bla\textsubscript{IMP} (Carapenem resistance). We found that 50, 30 and 3 from the 50 \textit{K. pneumoniae} strains by mPCR screening harbored \textit{wzc}, \textit{bla\textsubscript{CTX-M}} and \textit{bla\textsubscript{IMP}} respectively. Accordingly, mPCR clearly succeeded among other typing methods and proved to be a useful technique in distinguishing related and unrelated \textit{K. pneumoniae} clinical isolates. The ESBL-phenotype was associated with mPCR pattern B (table 4), although there were 2 isolates which were ESBL-positive by DDS and found belonging to mPCR pattern A, i.e. lack \textit{bla\textsubscript{CTX-M}} gene. Such controversy may be attributed to the possibility of ESBL production due to occurrence of other genes not amplified. Also, sometimes, multiple genes are responsible for production of ESBL in a single isolate.

On the other hand, there was a single isolate which was ESBL-negative by DDS, and its DNA amplification profile belonged to mPCR pattern B, i.e. possess \textit{bla\textsubscript{CTX-M}} gene. This may
be described by that the sensitivity of DDS test is less than the genetic method used.

Success of DNA amplification of the ESBL genes revealed that the \textit{K. pneumoniae} clone responsible for the outbreak harbored ESBL type \textit{bla}_{\text{CTX-M}}\textit{ group 1}. This is, to our knowledge, the first time to amplify such gene in our locality.

More interestingly, plasmid analysis was efficient to differentiate \textit{K. pneumoniae} strains; such method delineated 7 distinct plasmid profiles among the 50 studied strains. However, mPCR appeared less discriminatory, since it stated only 3 genotypic groups, probably due to aiming for detection of only 3 genes. Here, we should report that it is of great importance that reaction conditions, including DNA template concentration, annealing temperature and the choice of the primer pairs, should be standardized to avoid variations in mPCR patterns.

To conclude, phenotypic methods are only screening methods for detection of ESBL in a routine laboratory. The genotypic methods actually help to confirm the genes responsible for ESBL production. This method provided an efficient, rapid differentiation of ESBL in \textit{Klebsiella} and could be used as a rapid tool for epidemiological studies among ESBL isolates.

In conclusion, the present study demonstrated that \textit{K. pneumoniae} infections in MUCH have been caused by a variety of strain genotypes. It can help further efforts to understand the spreading of this bacterium.

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The complex differentiation of Klebsiella pneumoniae resistant organisms within the hospital environment.

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