Investigation of the prevalence and mechanisms of resistances of bacteria involved in respiratory tract infections

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PhD thesis

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INTRODUCTION

The most common infectious diseases are those involving the upper and lower respiratory tract. Their treatment are frequently difficult due different resistance mechanisms spreading among respiratory pathogens and leading to multi-resistant strains.

S. pneumoniae, the most common causative agent of community acquired pneumonia can be ranged in 90 different serotypes according to its polysaccharide capsule antigen. The mechanism of action of vaccines is based on the fact that antibodies produced against these antigens prevent invasive infections caused by the same serotype *S. pneumoniae*. Vaccines against severe *S. pneumoniae* infections were developed to cover the most prevalent serotypes.

Previously all *S. pneumoniae* strains were susceptible to penicillins. Penicillins act on penicillin binding proteins (PBPs) present in the cytoplasmic membrane of the bacteria and are involved in the peptidoglycan synthesis of the cell wall as transpeptidases and transglycolases. Penicillins irreversibly bind to the PBPs and inactivate them. The synthesis of the PBPs are coded by mosaic genes. Depending from the composition of these mosaic genes low-level or high-level penicillin resistance as well as amoxicillin and 3rd generation cephalosporine resistance can develop in pneumococci. Penicillin G is not the drug of first choice any more in *S. pneumoniae* infections, however the *in vitro* resistance against it indicate well the resistance of the strains to beta-lactam antibiotics. About 50% of the population harbour *S. pneumoniae* in the nasopharynx without any symptoms (carrier status). All virus infections promote the binding of bacteria to the epithelial cells of the respiratory tract. *S. pneumoniae* may spread from the nasopharynx and reach the lower respiratory tract causing inflammation. *S. pneumoniae* is still one of the most prevalent lower respiratory tract pathogens especially in the case of community acquired pneumonia. According to a surveillance data from the US, 50% of those died of severe *S. pneumoniae* infections could have been saved by the widespread vaccination against *S. pneumoniae*. Similar to other European countries Hungary has developed its own vaccination policy to prevent *S. pneumoniae* infections.

In ventilated and cystic fibrosis patients the biofilm formation plays important role in the pathogenesis of *P. aeruginosa* infections. Antibacterial agents can not cure these patients without the help of their own immune system. Bacteria living in biofilms are protected against antibacterials and sometimes even against phagocytes. According to this the decrease of the biofilm formation or destruction of the biofilm may help in the successful treatment of these patients. Certain macrolide antibiotics are proved to inhibit the alginate production of *P. aeruginosa* strains and therefore they are used as an adjuvants treatment in cystic fibrosis

patients. However, the treatment of infections caused by multi-resistant *P. aeruginosa* strains are often seemed as impossible.

The most common Gram-negative bacteria causing nosocomial pneumonia are *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Escherichia coli*, *Serratia marcescens* and *Pseudomonas aeruginosa*. As a result of misuse and overuse of beta-lactam antibacterials the beta-lactamase producing isolates and those producing extended spectrum beta-lactamases (ESBLs) can be isolated more and more frequently. The mortality rate of severe nosocomial pneumonia is between 50 and 100%, hence the prevalence and resistance patterns of pathogens should be monitored to be able to find optimal empiric therapy.

AIMS

Aims of this study were:

1. To evaluate the penicillin, amoxicillin, ceftriaxone and macrolide resistance of clinical *S. pneumoniae* isolates obtained between 1997 and 2005. Analyses were performed according to age groups, specimen types and specimen origin (inpatient or outpatient). We collected data on the carriage of *S. pneumoniae* in children. We were also interested in whether the *S. pneumoniae* vaccines introduced in Hungary cover the frequently isolated serotypes. We also investigated the efficacy of a new 3rd generation cephalosporin against high-level and low-level penicillin resistant *S. pneumoniae* isolates.
2. To follow the respiratory tract colonisation of cystic fibrosis (CF) patients treated in a cystic fibrosis outpatients clinic between 1999 and 2001. We aimed to develop an *in vitro* method suitable to detect the biofilm production of *P. aeruginosa* strains isolated from these patients and to test the inhibitory effect of macrolides on biofilm formation by the new method.
3. To compare and evaluate the clinical relevance of two lower respiratory tract sampling methods used in an Intensive Care Unit. The prevalence and ESBL production of *Enterobacteriaceae* strains isolated from patients with nosocomial pneumonia were also analysed.
4. To investigate the synergistic effect of different antibiotic combinations and to select a time-saving method to help clinicians in the selection of effective antibiotic combinations against multi-resistant respiratory tract pathogens.
5. To study the resistance mechanisms of common respiratory tract pathogens against beta-lactam antibiotics by conventional and molecular methods.

MATERIALS AND METHODS

Bacterial strains

Thirty-five *S. pneumoniae* isolates were obtained from the nasopharynx of healthy children (aged 3-6 years) attending day-care centres. 93 isolates were collected earlier in the Heim Pál Hospital, Budapest. All other *S. pneumoniae* isolates - involved in different studies - were isolated in the Institute of Clinical Microbiology, University of Szeged from the upper and lower respiratory tract, blood culture, and cerebrospinal fluid of symptomatic patients.

The *P. aeruginosa* isolates (110) were recovered from the upper and lower respiratory tract of cystic fibrosis patients. The 179 *K. pneumoniae*, 82 *E. cloacae*, 7 *E. coli* and the 15 *S. marcescens* isolates were cultured mainly from the lower respiratory tract of different patients.

Specimen collection

The nasopharyngeal swabs were used for the surveillance study (*S. pneumoniae* carriage), sputum and throat samples were taken from cystic fibrosis patients. Sputum was cultured after cytological analysis (acceptable sample: >25 neutrophils and <10 epithelial cells/ field; examining at least 40 fields with 100x zoom). The endotracheal aspirates (EA) - obtained from ventilated patient - were examined microscopically to determine the presence or absence of neutrophil granulocytes. In the case of EA, the cut-off value was $\geq 10^5$ colony forming unit/ml (CFU/ml), while in the case of protected bronchoalveolar lavage (miniBAL) it was $\geq 10^3$ CFU/ml.

Serotyping of *Streptococcus pneumoniae* isolates

Serotyping was performed with the *S. pneumoniae* typing antisera purchased from Mast Group Ltd (Bootle UK).

Antimicrobial susceptibility testing

Susceptibility testing was performed by the disk diffusion method. The minimum inhibitory concentration (MIC) was determined by Etest (AB Biodisk, Solna, Sweden), agar dilution or micro-broth dilution method. NCCLS/CLSI guidelines were used rigorously during the whole study.

Investigation of beta-lactamase production and its genetic background

Beta-lactamase production was detected by discs containing nitrocefin (Difco). The substrate profiles of the enzymes were determined by the micro-colorimetric method of Escamilla. For the enzyme induction experiments the disc diffusion method was used. The inducer antibiotics were cefoxitin and imipenem whereas the indicator antibiotics were cefuroxime, cefoperazone, cefotaxime and ceftazidime. For the screening of ESBL production the double-disc method (ceftazidime and amoxicillin/clavulanic acid), the five-disc method of Jarlier (ceftazidime, cefotaxime, aztreonam, cefepim and amoxicillin/clavulanic acid) or the Etest methodology was

applied. For the molecular investigation of ESBL production of *K pneumoniae*, *E. cloaceae* and *S. marcescens* strains the plasmid DNA was isolated by the magic Miniprep DNA Purification Kit (Promega Madison WI). Plasmids were detected in 0.7% agarose gel, stained with ethidium bromide. Specific primers for the TEM and SHV genes were used for the amplification. Single-stranded conformation polymorphism (SSCP) analysis was performed to differentiate different types of SHV genes after digestion by PstI enzyme. The fragments of the ssDNA were visualised by silver nitrate staining.

Examination of synergisms

To evaluate the combined effect of beta-lactamase inhibitors (clavulanic acid, sulbactam) and cefoperazone, the chequerboard titration (according to Lorian) and the killing curve method were used. The initial inoculum was 10^5 CFU/ml. The effect of cefoperazone alone (at 1xMIC or 2xMIC concentrations) or combined with 1 mg/l clavulanic acid and 1mg/l or 16 mg/l sulbactam was evaluated by killing curve method. The cultures were incubated at 37°C in normal atmosphere. The CFU counts were determined after incubation for 6 and 24 hours. The Fractional Inhibitory Concentration (FIC) index was calculated to classify the combined effect. The applicability of the Etest method in synergism studies was also evaluated.

Examination of biofilm production

The biofilm production of mucoid *P. aeruginosa* strains isolated from the respiratory tract specimens of cystic fibrosis patients was investigated *in vitro* by the Enzym-Linked Lectinsorbent Assay (ELLA) according to Leriche. After standardisation of the method we investigated the inhibition effect of clarithromycin on biofilm production (on those strains which proved to be good biofilm producers). In this experiment the method of Leriche was changed as clarithromycin (to produce a solution with 50, 100, 200, 300, and 400 mg/l final clarithromycin concentrations) was solved in the culture medium. After the daily washing procedures with the clarithromycin buffer we added peroxidase conjugated lectin and compared the inhibitory effect of clarithromycin to control cultures.

Direct Etest examination of sputum

After cytological examination and mucolysis, 100 µl of the sputum of cystic fibrosis patients were plated on blood agar. Etest strips containing antipseudomonal drugs were placed on the culture plates and incubated for 18-24 hours at 37 °C in normal athmosphere. We determined the MICs of different *P. aeruginosa* strains, revealed the presence of multiresistant strains and examined the susceptibility of the normal flora to the corresponding antibiotics.

Antibiotic consumption

The annual wholesaler data on the community pharmacy sales of antibacterials were collected retrospectively for the 8-year period 1998-2005. Products were classified and calculations were performed according to the 2005 version of the World Health Organization ATC (Anatomical -

Therapeutic - Chemical) DDD (Defined Daily Dose) index. For investigating the effect of antibiotic consumption on penicillin and macrolide resistance of *S. pneumoniae*, only the oral formulations were included in the analyses (beta-lactam antibacterials [ATC code: J01C] and the macrolides, lincosamides [J01F]). The nationwide antibiotic consumption and that of Csongrád County were expressed as DDD per 1000 inhabitant-days.

Statistical analysis

All resistance data were collected retrospectively from our laboratory database. Repeat isolates from individual patients were excluded. Differences in the distribution of resistance patterns were analysed by means of chi-square test and Fisher's exact test, as appropriate, using the SPSS program package (version 13.0). A P value of <0.05 was considered to be statistically significant.

RESULTS

1. Results obtained during investigations of *S. pneumoniae* isolates

Due to controversial data published in the '90s about the penicillin resistance of *S. pneumoniae* isolates in Hungary, a retrospective evaluation of penicillin, amoxicillin, ceftriaxone and macrolide resistance of *S. pneumoniae* isolates was carried out. All clinical isolates between 1998 and 2005 were retrieved from the database of our institute. During this period the resistance determination methods suggested by NCCLS/CLSI were used rigorously. Until 2002 our data showed a much lower prevalence of high-level penicillin resistant *S. pneumoniae* isolates compared to those published by other authors and could be found in national databases. These discrepancies might be due to the usage of different, not standardised methods in the screening and confirmation of high-level penicillin resistant pneumococci in many Hungarian laboratories.

During this 8-year period both inpatient and outpatient samples were included, but the proportion of the patients and hence the number of the isolates in these two groups had changed over time. Between 1998 and 2001 (Period I) our laboratory provided a service almost exclusively for the different clinical wards and for their outpatient departments, while from 2002 (Period II) increased number of samples were sent by general practitioners and from the recently merged general paediatric hospital. These changes resulted in an increase in the total number of *S. pneumoniae* strains isolated from outpatients, especially from children. During the 8-year period 2670 *S. pneumoniae* strains were isolated from different samples. 1267 strains were derived from inpatients, great majority (983 strains) from children (age: $\leq$14 years). The frequency distribution of strains with high-level or low-level penicillin resistance varied considerably between the two periods. The prevalence rate of high-level penicillin-

resistant *S. pneumoniae* strains decreased from Period I to Period II, despite of the same detection methodologies that were used. Among the 0-2 year old children, both in inpatients and outpatients, the prevalence rates of high-level penicillin-resistant *S. pneumoniae* strains were considerably lower in Period II compared to Period I (20% vs. 4% and 21% vs. 2%, respectively). A similar trend was seen among the strains isolated from 3-14 year old patients. Among the isolates obtained from adults (>14 years), the prevalence rate of high-level penicillin-resistant strains were low in both periods (8.7% vs. 2.5%). The amoxicillin and ceftriaxone resistance was rare during the whole study. The erythromycin and clindamycin resistance of the isolated strains were continuously and uniformly high.

The national penicillin (J01C) consumption remained relatively stable between 1998 and 2005 (mean ± SD: 8.45±0.51 DDD per 1000 inhabitant-days), with a transient peak in 1999, possibly in consequence of an influenza outbreak with concomitant bacterial infections. The consumption of penicillins with extended spectrum (J01CA) gradually decreased, while the usage of penicillins combined with beta-lactamase inhibitors (J01CR) continuously increased. The consumption of the J01C, J01CA and J01CR subgroups in Csongrád County exhibited similar trends and were consequently slightly higher compared to national values. The consumption of macrolides and lincosamides (J01F) rose in Hungary through the years, as well as in Csongrád County. The antibiotic consumption and the prevalence rate of penicillin resistant *S. pneumoniae* strains are not associated.

Sero-types of 57 randomly selected *S. pneumoniae* isolates were determined. The sero-types of the strains recovered from 0-2 year old children were identical in 95% with those present in the conjugated pneumococcus vaccine used for the immunisation of this population. The isolates obtained from children above 2 years of age showed identity in 97% with the the serotypes of the corresponding vaccine.

We also collected nasopharyngeal samples from 195 healthy children attending 4 different day-care centres (two situated in the centre of the city and two situated outside of the city). Thirty three percent of them were *S. pneumoniae* carrier. An interesting observation is that children attending day-care centres in the city centre harboured *S. pneumoniae* only in 11%, whereas in those attending suburban day-care centres the carriage rate was 29%. Of the 35 strains, only one had an MIC of 4 mg/L for penicillin and an MIC of 1 mg/L for amoxicillin. An additional 13 strains had an MIC for penicillin between 0.125 and 1 mg/L, corresponding low-level penicillin resistance. Penicillin resistance was more frequent among children attending day-care centres in the city centre, presumably due to more frequent antibiotic use. None of the *S. pneumoniae* strains were resistant to ceftriaxone. Fourteen strains were resistant to erythromycin and clindamycin as well.

We also investigated the *in vitro* activity of cefditoren-pivoxil, a new 3rd generation cephalosporine against 35 isolates obtained from adult patients with pneumonia and against 93 *S. pneumoniae* strains recovered from children with upper respiratory tract infection. In this later group the prevalence rates of high-level and low-level penicillin resistant *S. pneumoniae* strains were very high (54% and 22%, respectively). The MIC values for cefditoren-pivoxil were much lower than the average serum levels obtainable with the regular doses. According to these, cefditoren-pivoxil could be a useful alternative in the treatment of infections caused by high-level or low-level penicillin resistant strains.

2. Results obtained during the investigation of *P aeruginosa* strains isolated from samples of cystic fibrosis patients.

Twenty four patients - treated in a special CF outpatient clinic in Szeged - were monitored between July 1999 and June 2001. The upper and lower respiratory tract samples were cultured to detect the appearance or continuous presence of potential respiratory tract pathogens. During this period *P. aeruginosa* was isolated most frequently. In 1999 38%, in 2000 33% and in 2001 36% of the patients were colonised with *P. aeruginosa*. This means that there was a permanent risk for colonisation and infection caused by *P. aeruginosa*.

An *in vitro* model was developed to detect the biofilm formation of *P. aeruginosa* isolates. Mucoid colonies - isolated from cystic fibrosis patients - were selected for these experiments. To standardise the method we determined the optimal duration of the biofilm formation, the culture conditions and the signal system. In this model it was possible to measure and quantify the biofilm formation inhibition effect of clarithromycin. During the selection of proper drugs to treat already colonised cystic fibrosis patients, it is mandatory to differentiate various *P. aeruginosa* isolates with different resistance patterns. A new culture technique and a direct antibiotic resistance determination method were introduced. We applied this method to the lower respiratory tract specimens of symptomatic cystic fibrosis patients in order to determine the most suitable antibiotic treatment to kill mucoid *P. aeruginosa* and to save the normal respiratory tract flora. This method was also suitable for the direct differentiation of colonising bacteria with diverse antibiotic resistance patterns.

3. Prevalence of the ESBL producing *Enterobacteriacae* strains isolated from the lower respiratory specimens of different patients.

We evaluated the clinical relevance of culture results obtained by different lower respiratory tract sampling methods. We confirmed the advantages of protected bronchoalveolar lavage (miniBAL) over the tracheal aspirates. The culture results of lower respiratory tract specimens obtained between July 1997 and October 1999 by the miniBAL method and by tracheal

aspirates were compared. During this period 63 patients had hospital acquired pneumonia. Out of the 95 miniBAL samples 49 were positive and overall 66 pathogens were isolated. When the culture results of the tracheal aspirates were compared to those obtained by the miniBAL identical species isolation was found only in 66%. We can conclude that the invasive sampling method (miniBAL) can not be replaced by the tracheal aspirate in case of the intubated patients with hospital acquired pneumonia as prolonged hospital stay may result in colonisation of the upper respiratory tract with bacteria and may lead to false isolation results with tracheal aspirates.

Between 2002 and 2005 the resistance patterns of Gram-negative pathogens isolated from the lower respiratoty tract of patients with nosocomial pneumonia were evaluated with special emphasis on resistance to beta-lactam antibiotics. The extended spectrum beta-lactamase (ESBL) production was tested by different detection methods. The number of the ESBL producing Gram-negative strains did not differ significantly during the study period, and no dominant ESBL producing strain was found.

4. Investigation of antibiotic synergisms in the case of Gram-negative respiratory tract pathogens.

During the routine *in vitro* testing of antibiotic resistance by disc diffusion method a synergistic effect was observed between cefoperazone and amoxicillin/clavulanic acid. For further investigation of this phenomenon chequerboard titration and killing curve methods were used to investigate the combined effect of cefoperazone-clavulanic acid as well as cefoperazone-sulbactam combinations against 7 *E. coli* and 9 *K. pneumoniae* strains producing different types of beta-lactamases (TEM and SHV). The effect of the cefoperazone/clavulanic acid combination was superior to that of cefoperazone/sulbactam, so this could be a suitable combination in the treatment of infections caused by beta-lactamase (TEM or SHV-type) producing Gram-negative bacteria.

110 clinical *P. aeruginosa* isolates were evaluated by the Etest methodology to find effective antibiotic combinations. Thirty isolates proved to be ciprofloxacin resistant of which 26 were multi-resistant (resistant to $\geq$3 drugs with antipseudomonal activity). According to the FIC index the ceftazidime/ciprofloxacin and piperacillin/amikacin combinations were synergistic against 10 strains, while the piperacillin/ciprofloxacin combination was synergistic against 7 strains. In case of ciprofloxacin-intermediate resistant or ciprofloxacin susceptible strains these synergistic effects were less frequently found. Our results show that Etests are very practical for routine everyday use as rapid *in vitro* data can guide the clinicans to find effective antibiotic combinations for the treatment of infections.

5. Investigation of resistance mechanisms of beta-lactam-resistant Gram-negative bacteria belonging to the Enterobacteriaceae group

Clinical isolates of *K. pneumoniae* (170), *E. cloaceae* (82) and *S. marcescens* (15) were investigated for the beta-lactamase production. Out of the 267 isolates 109 (41%) harboured one or more beta-lactamase genes. The most prevalent ESBL-types among the *K. pneumoniae* strains were SHV-2 and SHV-5, whereas among the *E. cloaceae* strains the inducible Class C beta-lactamase was detected in 55%. The *S. marcescens* strains - isolated from a nosocomial epidemy (12 clinical and 3 environmental isolates) - were investigated by molecular methods. The PCR-SSCP analysis suggested that 14 *S. marcescens* strains not only produced the Class C, inducible chromosomal beta-lactamase, but also acquired a plasmid-mediated SHV-2 type ESBL. The in vivo transfer of SHV-2 gene was assumed from an SHV-2 positive *K. pneumoniae* strain present simultaneously in the same patient. In the hospital environment, mostly in the case of respiratory tract infections, we have to take into consideration the accumulation of multiple resistance mechanisms coded by chromosomal or plasmid genes and the inter-species gene transfer.

CONCLUSIONS

S. pneumoniae, the most frequent causative agent of community acquired pneumonia and upper respiratory tract infections may have severe, life-threatening consequences. Our retrospective evaluation of penicillin, amoxicillin, ceftriaxone, and macrolide resistance of *S. pneumoniae* strains showed that the prevalence of high-level penicillin resistant strains was very low (5.58%) especially in adult patients (4%) and among invasive isolates (3.8%). Low-level penicillin resistance were 37% and 39% in the isolates obtained from inpatients and outpatients. Throughout this 8-year period we rigorously used internationally accepted methodologies for the screening and confirmation of resistances. These data differ greatly from those reported previously in Hungary. Resistance to amoxicillin (that is extensively used for the treatment of *S. pneumoniae* infections) and to ceftriaxone (that is used for the treatment of severe invasive infections) were 2.62% and 1.12, respectively. However the macrolide resistance was continuously high during the study period, which means that macrolides can not be used for the empiric treatment of *S. pneumoniae* infections.

The carriage rate of *S. pneumoniae* in children attending day-care centres was 33%. Resistance to penicillin, amoxicillin, ceftriaxone and macrolides was similar to that found among clinical isolates.

The investigation of the serotypes of randomly selected clinical *S. pneumoniae* isolates showed that 95% and 97 % of the serotypes were covered by the respective vaccines used for the immunisations. According to this, immunisation programmes may prevent severe consequences of *S. pneumoniae* infections in Hungary.

Our study shows that the new 3rd generation cephalosporine, cefditoren-pivoxil is very active against both high-level and low-level penicillin resistant *S. pneumoniae* strains. The MIC values of cefditoren-pivoxil were much lower than the average serum levels obtainable with the regular doses. According to these cefditoren-pivoxil could be a useful alternative for the treatment of infections caused by high-level or low-level penicillin resistant strains.

With the development of an *in vitro* method we were able to detect the biofilm formation of *P. aeruginosa* strains that are frequently colonising the airways of cystic fibrosis patients. With this method we could provide useful information about the inhibitory effect of macrolides (clarythromycin) on biofilm formation. By using the direct antibiotic resistance detection method - carried out with Etests - we can help to find the most appropriate antibiotics that are able to kill the simultaneously detected pathogen colonies and strains with diverse antibiotic resistance patterns while preserving the normal flora.

Gram-negative bacteria belonging to the *Enterobacteriaceae* group are the most common pathogens of ventilator-associated pneumonia acquired in intensive care units. To get clinically relevant culture results, the usage of miniBAL instead of tracheal aspirate is recommended in case of intubated patients. Screening the ESBL production of respiratory tract pathogens helps to select effective empiric therapy for these patients. The appearance of ESBL producing strains urge the restrictive use of certain beta-lactam antibiotics. As ESBL producing Gram-negative bacteria were rarely isolated in our intensive care units it can be regarded as a success.

In vitro methods are available to investigate the synergistic effect of antibiotic combinations. We confirmed that synergistic effect of cefoperazone and clavulanic acid against beta-lactamase producing Gram-negative bacteria is superior to the cefoperazone-sulbactam combination. The chequerboard titration and the killing curve method used for these examinations are time- and labour-consuming methods. A more suitable method for the every day routine, the Etest methodology was evaluated and compared to the classical methods. The synergistic effect of different antipseudomonal antibitotic-ciprofloxacin combinations were proved by this method even in case of multi-resistant *P. aeruginosa* strains. The efficacy of the combinations (FIC index) was highly dependent on the isolate, on the level of the resistance and on the combined antibiotics. Individual testing is necessary to guide therapy caused by multi-resistant strains.

To investigate the genetic background of beta-lactam resistance is extremely important as beta-lactamase genes present on plasmids may easily spread in the hospital environment. On the other hand chromosomal genes as well as plasmid genes mutate according to the antibiotics used in the wards. Our data confirm the widespread presence of TEM and SHV genes among clinical Gram-negative isolates. The high co-prevalence of the inducible chromosomal and stable derepressed beta-lactamase genes in *E. cloaceae* was also confirmed. In a nosocomial epidemy the in vivo transfer of SHV-2 gene was assumed from an SHV-2 positive *K. pneumoniae* strain into a *S. marcescens* strain producing a Class C beta-lactamase. The presence of both strains could be confirmed in the same ward, in the same patient and at the same time.

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PUBLICATIONS

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