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PROBLEMS OF INFECTIOUS AND PARASITIC DISEASES
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NEUTROPHIL RESPIRATORY BURST ACTIVITY IN NEONATAL INFECTION

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SUMMARY

To determine the neutrophil respiratory burst activity (NRBA) in neonates with infection, and correlate the obtained results with the IL-6 levels. Thirty-nine neonates (23 preterm and 16 term) were studied. They were: neonates with infection and positive blood culture (n=20), neonates with infection and negative blood culture (n=10) and neonates without infection, as controls (n=9). Group of 21 healthy adults were studied, too. The NRBA was measured in whole blood flow cytometric assay. The test was performed without stimulation and with stimulation with fMLP. IL-6 levels were determined by ELISA. Unstimulated neutrophils of the control neonates without infection demonstrated greater NRBA, compared with the healthy adults (P <0.05). After fMLP stimulation control neonates had similar results to adults. Nonstimulated burst activity in the neonates with infection (with positive blood culture, as well as with negative blood culture) was significantly higher than those in control neonates (P<0.05). After fMLP stimulation, the neonates with infection had also elevated NRBA, but the difference was statistically significant only in neonates with negative blood culture (P<0.05). The IL-6 were elevated in neonates with infection (with positive and with negative blood culture (P<0.01), but there is no correlation with the NRBA. These data demonstrate that in neonates with infection (with positive and with negative blood culture) NRBA was elevated, compared with the noninfected neonates, with an exception of the group with positive blood culture after fMLP stimulation. There is no correlation between the NRBA and elevated IL-6 levels.

Key words: neutrophil respiratory burst, dihydrorhodamin 123-flow cytometry, IL-6, neonates, infection.

Neutrophils are phagocytic cells whose function is to play a prominent part as a first line defense against bacterial infections. Upon activation with appropriate stimuli normal phagocytes exert their microbicidal effect by developing a reaction, known as respiratory burst (oxygen-dependent intracellular killing). This reaction is characterized by the generation of reactive oxygen species including superoxide anions, hydrogen peroxide and hydroxyl radicals (1).
In both full-term and preterm neonates, there is an increased risk of severe bacterial infections /2/. Immaturity in neonatal host defense and impairment of neutrophil function may be responsible for this risk (3). As the early diagnosis of neonatal infection is a difficult task, many studies have searched for new diagnostic methods. In this aspect, the changes of neutrophil respiratory burst activity (NRBA) in infants may be useful as an early parameter of infection.

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ABBREVIATIONS USED IN THIS PAPER: NRBA - Neutrophil R0espiratory Burst Activity

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The current literature does not present a lot of data about the ability of neonatal phagocytes to generate oxidative burst activity in infection. There are many conflicting results. Defective oxidative metabolic responses of neutrophils were reported in some of these studies (4, 5, 6). Others failed to confirm this data and demonstrated increased neutrophil response or neutrophil function near to that of adults (7, 8, 9). The controversial results may be due to variations in used methods (NBT test, chemiluminescence or various flow cytometric test methods), types of phagocytes studied (polymorphonuclear cells, monocytes or macrophages), or type of stimulating agents (E.coli, PMA, fMLP) (4, 7, 10). Also, many neonatal characteristics (type of delivery, gestational age, prematurity) or used drugs for therapy seem to have effects on ability of neonatal phagocytes to generate oxidative burst in infection (4, 7, 11, 12, 13, 14, 15).
The aim of this study was to investigate the NRBA in neonates with infection. It was made by a whole blood flow cytometric analysis with an oxidative probe dihydrorhodamin 123 (DHR). Obtained results from NRBA were correlated with the IL-6 levels, because it is important parameter in the early diagnosis of neonatal infection and has been proven to play a role in the inflammation, acute phase reactions and neutrophil function (16, 17, 18).

PATIENTS AND METHODS

Study population. All studied neonates were admitted to the neonatal intensive care unit at the University Pediatric Hospital in Sofia, during a six-month period. On the basis of clinical symptoms and laboratory parameters, the 39 neonates were retrospectively assigned to three groups.
Group1: Group of 20 neonates with positive results on blood culture, abnormal laboratory parameters (leukopenia less than 5x103/mm3 or leukocytosis more than 25x103mm3, trombocytopenia, and CRP>10g/l) and characteristic clinical symptoms of infection. (19). These neonates were classified as having sepsis. They were12 preturm (GA=33.9 _ 3 weeks, BW = 1, 7 _ 772 g) and 8 term (GA = 40 weeks, BW = 3, 27 _ 524 g). Gram-positive bacteria (Staphylococcus aureus = 5, Streptococcus epidermidis = 4) were isolated in 9 cases, and a Gram-negative bacteria (Escherichia coli = 4, P. aeruginosa = 6) were isolated in 10 cases. One infant had a blood culture with Candida albicans.
Group 2: Group of 10 neonates with infection, but negative results on blood culture. Suspicion of infection was based on clinical signs and the same laboratory parameters - abnormal leukocyte and trombocyte count and CRP levels. The neonates were 4 term (GA = 40, 3 _ 0.6 weeks, BW = 2.78 _ 202 g) and 6 preturm (GA = 32 _ 3 weeks, BW = 1, 55 _ 402 g.
Group 3 (Control neonate group): Group of 9 noninfected neonates (no clinical or laboratory signs of infection), 4 term (GA = 39.8 _ 0.4 weeks, BW = 2.95 _ 248 g) and 5 preturm (GA=34, 5 _ 2 weeks, BW = 1, 95 _ 450 g). These neonates were admitted to the unit for reasons other than infection.
The measurements were performed once in each neonate at admission in the same blood specimens taken for routine laboratory procedures. Informed consent from the parents was obtained according to the rules of the ethical committee. The studied neonates were tested in first 10 days of their life. All of them were treated with antibiotics.
Control group of healthy adults: Healthy adults (n= 21) were used as a daily positive and negative control of the flow cytometric test and compared with healthy neonates.
Flow cytometric oxidative burst assay - Burst test. Heparinized blood samples were collected and tested within 1 h after bleeding. The quantitative determination of leukocyte

Table. 1. Neutrophil respiratory burst activity (% activated cells) in neonates with infection, measured by DHR 123 flow cytometry.

Burst test	Group 1 n = 20	Group 2 n = 10	Group 3 n = 9	P1	P2
Non-stimulated					
Median	9.53	12.17	4.94	0.0496	0.0185
Range	(1.57 - 28.71)	(3.38 - 16.38)	(0.44 - 9.65)		
fMLP-stimulated					
Median	12.58	15.47	9.06	0.1282	0.0431
Range	(5.50 - 39.95)	(8.98 - 47.33)	(1.77 - 20.93)		

P1 - Significance between neonates with positive blood culture (group1) and neonates without infection (control group 3) ($p<0.05$ as significant).
P2 - Significance between neonates with negative blood culture (group2) and neonates without infection (control group3) ($p<0.05$ as significant).

oxidative burst was done by BURSTTEST kit, ORPEGEN, Pharma, according to the instructions of the kit. Briefly, heparinized whole blood was mixed with PBS - nonstimulated burst test or with fMLP (N-formil-methionyl-leucyl-phenylalanine) for low physiological stimulation - stimulated burst test. After that, the blood was incubated for 10 min at 37°C in a water bath. The oxidation was made by adding substrate solution (DHR 123); then incubation (10 min at 37°C) followed. Reaction was stopped by adding lysing solution (2 ml for 20 min at room temperature). Once, the samples were washed with PBS and resuspended with PBS containing propidium iodide (10 min at 0°C) for DNA staining. DHR is freely permeable, localizes in the mitochondria and after oxidation by H_2O_2 and O2- to rhodamine 123, emits a bright fluorescent signal.

Flow cytometry. A FACS Calibur flow cytometer (Becton Dickinson) was used for acquisition and analysis of the data. To exclude cell debris and platelet aggregates from analysis, a gate was set on propidium iodide-stained leukocytes during acquisition in the red fluorescence (575 nm, FL 2 channel). Granulocytes were identified by forward light scatter and side scatter (Fig.1A). For each measurement, 10 000 events were collected in the granulocyte gate. Rhodamine 123 green fluorescence was collected in the FL1 channel and a fluorescence histogram was plotted (Fig. 1B). For the analysis of reactive oxygen species production, the shift to the right in the green fluorescence (530 nm, Fl1) was determined. The results were recorded as the percentage of the neutrophil fluorescent cells (% positive cells). The discrimination for negative/positive fluorescence was set by daily examination of the histogram of the negative adult controls (nonstimulated test with less than 1% of neutrophils reacting). The adult controls were stimulated with E. coli for optimal in vitro stimulation, where the percentage of activated cells was more than 96%.

Measurement of IL-6. Commercially available enzyme linked immunoassay kit OptEIATM Set, PharMingen; specific for IL-6 was used to assess the concentration of this cytokine. The minimal detectable level was 4 pg/ml. IL-6 was measured in the serum taken at the same day as for the burst test. All samples were tested in duplicate. Peripheral blood samples were centrifuged, and serum was stored at -60°C until assayed.

Statistical analysis. The Mann-Whitney test was applied, as appropriate, to analyze differences among groups. The results were expressed as medians with the corresponding ranges. SpearmanЛЬs correlation coefficient (r-value) was used to correlate the NRBA with levels of IL-6. Results from GA and BW were presented as mean _ SD. All the analyses were two-tailed and differences with a P value < of 0.05 were considered statistically significant.

RESULTS

Neutrophil respiratory burst activity in the neonates without infection (controls) and adults. The percentage of DHR 123-positive neutrophils (nonstimulated burst test or baseline oxidative activity) in the neonates without infection was median= 4.94%, range =0.44-9.65%. The oxidative activity

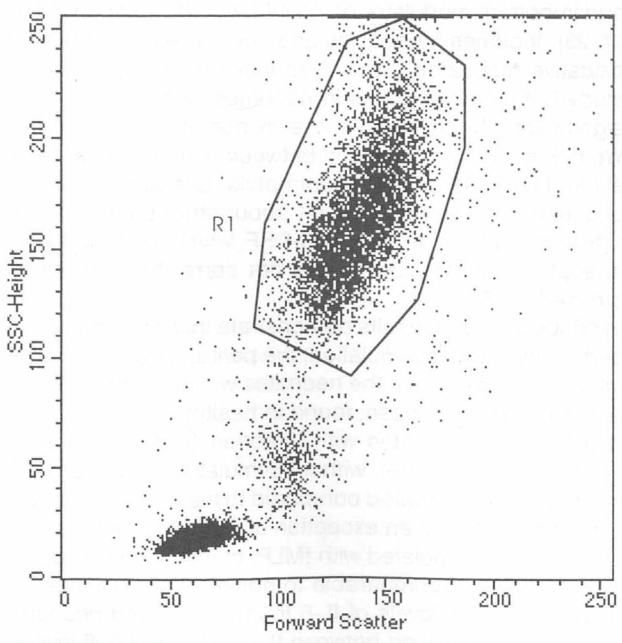

Figure 1A. Flow cytometric dotplot (forward scatter and side scatter) resulting from the analysis of blood sample of a healthy adult person. Neutrophils are presented as R1 gate.

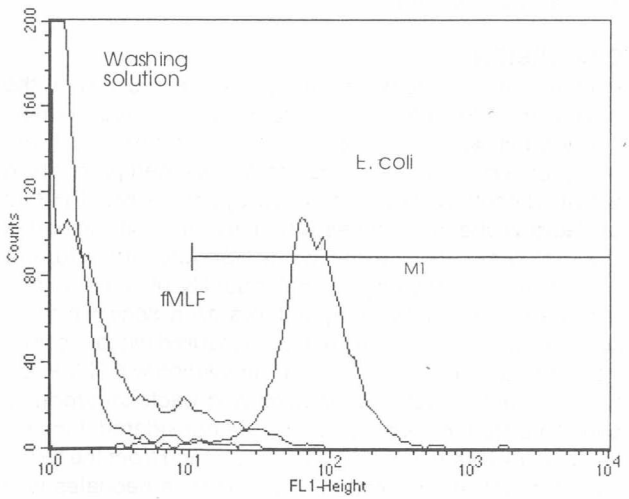

Figure 1 B. Histogram of relative fluorescence intensity expressing the percentage of oxidizing neutrophil cells (dihydrorhodamine -123 positive cells - FL1) from healthy adult control. Washing solution (nonstimulated cells), fMLP, and E. coli assays are simultaneously shown together with the marker M1 to delineate a region of positive cells.

after fMLP stimulation in this group was median=9.06%, range =1.77 - 20.93%.

The oxidative activity of daily controls (healthy adults) without stimulation and after fMLP stimulation was median= 1.72%, range= 0.07-4.52% and median= 9.27%, range 3.16-22.9%. Compared with adult controls, neonates without infection had higher NRBA of nonstimulated cells ($P < 0.05$) and similar NRBA of fMLP stimulated cells .

Neutrophil respiratory burst in the neonates with infection. The results from burst testing of neonates are presented in Table 1. When the burst test is performed without stimulation, the percentage of DHR 123-positive neutrophils of the neonates with positive blood culture (median=9,53%, range= 1.57-28.71%), as well as of the neonates with negative blood culture (median=12.17%, range= 3.38-16.38%) were significantly higher than the values found in the non-infected control neonates (median=4.94%, range= 0.44-9.65%, $P<0.05$).

When the burst test is performed with fMLP stimulation, the percentage of DHR 123-positive neutrophils was significantly higher in neonates with negative blood culture, compared to the controls (median=15.47%, range 8.98-47.33%, vs. median=9.06%, range=1.77-20.93%, $P<0.05$). After fMLP stimulation, there was a trend toward a greater percentage of activated neutrophils in the group of neonates with positive blood culture (septic neonates), but the difference was statistically nonsignificant when compared with control group (median= 12.58%, range 5.50-39.95% vs. median=9.06%, range=1.77-20.93%, $P>0.05$).

A decreased capacity for oxidative activity of neutrophils was demonstrated in one case (premature newborn infant of 35 weeks with S. aureus severe sepsis) where two measurements of NRBA had been made. The percentage of activated neutrophil cells (non-stimulated and stimulated burst test) was at admission 6.48% and 23.28%. After 10 days NRBA was further extremely depressed (0.54% and 1.99%) and 24 hours later the baby died of septic shock (the results from second measurements are not included in Table 1).

Cytokine levels. The serum IL-6 concentrations in the neonates with positive blood cultures (median=165 pg/ml, range=0-1800pg/ml) and with negative blood cultures (median=210 pg/ml, range=32-630pg/ml) were significantly higher than in control group (median=32 pg/ml, range=0-61pg/ml, $P<0.01$). The percentage of DHR 123-positive neutrophils of the neonates with positive and negative blood cultures did not correlated with the levels of IL-6, measured at the same time ($r=0.025$, $p=0.9091$).

DISCUSSION

In recent years increasing interest has been shown in the evaluation of PMN function in neonates. Neutrophil respiratory burst activity in neonates with infection is still not well explored. To examine the reactive oxygen products in activated neutrophils we used flow cytometry with DHR as oxidative probe in neonates with infection. The method is technically simple and follows a standard procedure; it can be performed using a small quantity of whole blood, and is considered by many authors as a sensitive functional assay (4, 7, 20). NRBA was measured without stimulation and after low physiological stimulation with fMLP. On the basis of the fact, that bacteria and bacterial products can stimulate the immune system we considered it important to analyze separately the data obtained from the blood culture-negative and blood culture positive neonates with infection.

Our results demonstrated that the NRBA of the control neonates without signs of infection (non-stimulated burst test) was higher than those, found in healthy adults. After fMLP low physiological stimulation the results from NRBA of neonates are similar to NRBA of adults. Using nonstimulated burst test, we found out that the NRBA of neonates with infection (with positive blood culture, as with negative blood culture) were higher compared to that of non-infected control neonates. Priming of the cells with fMLP resulted in enhanced DHR oxidation in neonates with negative blood culture compared with controls. Neonates with positive blood culture (neonates with sepsis) demonstrated NRBA, which was also high, but the increase was not statistically significant, compared with controls. These results suggest that increased NRBA might be caused by infection; however in more severe cases, such as sepsis, this activation is not so prominent. And as a consequence of prolonged bacterial antigen stimulation, functional exhaustion of neutrophil cells and their diminished oxidative activity may be observed (21). For example, one infant with S. aureus sepsis had very low percentage of activated neutrophils cells in nonstimulated and stimulated burst test in the day before the death. Similar to our results (using the same assay kit and the same fMLP stimulation) have been reported by Gessler et al (7). The authors found an increased percentage of DHR 123-positive neutrophils in neonates with increased levels of C-reactive protein compared to neonates without infection. Also, they found lower NRBA in healthy adults, compared with neonates without infection. The authors suggested that determination of respiratory burst might prove to be a new laboratory parameter of neonatal infection.

On the contrary, Drossou at al. (4) using a whole blood flow cytometric microassay with DHR 123, but stimulation by PMA (optimal stimulation), found out that NRBA was depressed in the stressed and septic neonates (term and preturm). Defective oxidative metabolic responses and stimulus-specific abnormality in the respiratory burst activity of neutrophils from stressed neonates was suggested by Shigeoka at al., too (5, 6). Some authors showed also, that NRBA of neonates without infection is comparable to that of adults (3, 4, 5, 9). The discrepancies of the results may be due to the use of different methods, severity of the infection, prematurity, gestational age, type of stimulation, or type of studied material (separated lymphocytes or whole blood) (4, 7, 22).

IL-6 is a highly sensitive marker of infection in the neonates and important modulator of the inflammatory response (16, 17, 23). It primes monocytes and neutrophils for enhanced oxidative respiratory burst responses in vitro (24). In our study IL-6 concentrations in neonates with infection were significantly higher than those in noninfected neonates. We did not find a correlation between results from IL-6 levels and DHR-123 positive neutrophils. Similar results were obtained by Drossou at al. (4) about other proinflamatory cytokines - TNF-α, IL-1β and G-CSF, which were increased in septic neonates, but this is not correlated with an increased NRBA.

In conclusion, our results demonstrate that when the test is performed without stimulation, the percentage of DHR 123-positive neutrophils of the neonates without signs of infection is higher than those, found in healthy adults.

The NRBA in neonates with infection (with positive and negative blood culture), without stimulation and after fMLP stimulation was elevated compared to the noninfected neonates controls, with an exception of the group with positive blood culture stimulated with fMLP. In that case the results were higher, but comparable to noninfected neonates.

Despite the high levels of IL-6 found in infected neonates there is no correlation between the NRBA and IL-6 levels. Although it is difficult to make a comment from such a small number of patients, it can be suggested that NRBA seems

to be a valuable parameter as an additional diagnostic test for early-onset bacterial infection in neonates. More investigations are required to clarify its role in clinical practice.

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PREVENTION OF SURGICAL SITE INFECTION INSTITUTIONALLY - PROJECT PHASE I

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SUMMARY

Surgical site infections (SSI) constitute an important group of nosocomial infections. In aim of reducing their rate at our institute, several important steps have been undertaken. After consulting the contemporary literature, we re-enforced the Infection control Committee and created an Antibiotic policy Committee within the Drug Therapeutic Commission. The Surgery department was renovated, we prepared Institutional programs for Infection control and Rational antibiotic policy. We introduced an audit of antibiotic prescriptions, both for Antibiotic prophylaxis in surgery, and therapeutic antibiotic usage. The rate of SSI was stable < 3-5 % during the last 3 years.

Key wards: surgical site infection, antibiotic prophylaxis, audit of antibiotic prescriptions

Surgical Site Infections rank second/third top place among nosocomial infections, with an Incidence rate varying from 0.5 to 15 % (1-6, 8, 9). Besides representing the most costly nosocomial infections, they account for significant higher mortality, disability and emotional stress.

Our institute: Medical Institute - Ministry of the Interior, is a 350-bed multiprofile hospital with specialized surgical departments. The rate of patients admission/year is ~ 7790.

In 2002 we began a project, aimed at reduction of surgical site infections (SSI).

MATHERIALS AND METHODS

The project started with a literature review.

The contemporary literature and published Guidelines were reviewed, incl.:

- HICPAC/CDC's "Guideline for Prevention of Surgical Site Infection, 1999"
- WHO's "A practical guide "Prevention of hospital-acquired infections", 2nd edn, 2002
- ISID's "A Guide to Infection Control in the Hospital", 2nd edn, 2002
- Zanetti & Platt "Guidelines for Perioperative Antibiotic Prophylaxis"

Although a Hospital Infection Control Committee had been already settled, we included new members and elaborated an up-dated Hospital Infection Control Program.

Another important step consisted in the creation of Hospital Antibiotic policy Committee within the Hospital Drug Therapeutic Committee.

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ABBREVIATIONS USED IN THIS PAPER: SSI, Surgical site infection; SICU, Surgical Intensive Care Unit; DDD/ 100 bed-day, defined daily doses/100 bed-day; MRSA, methicillin-resistant Staphylococcus aureus; ESBL, extended-spectrum beta-lactamase; GNNF, Gram-negative non-fermenters

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Decision was made to perform a 3-phase project: Prophylaxis of Surgical Site Infections:

* Phase 1. Audit of current Infection Control and Antibiotic prescribing practices, analysis and design of measures to improve the strategies
* Phase 2. Implementation of the improved strategy
* Phase 3. Evaluation of the results of intervention

RESULTS

One of the most important achievements in Phase 1 project until now is the reconstruction and updating of the surgical department according to the new standards. Architecture plans were developed and as a result of their realization the main building was renovated - patients rooms, manipulation rooms have been re-constructed, hygienized and re-furnished. Financial constrains did not allow, however, to fully modernize the operating theatre, e. g. ensuring the necessary positive air pressure in the operating rooms, nor ventilation with 15 to 25 changes of filtered air per hour. Both laminar airflow and HEPA filters would not be possible in near time.

Surveillance of nosocomial infection is performed both by direct prospective surgical observation (surgeon, nurse) and indirect monitoring by Microbiology Laboratory. This surveillance emphasizes inpatients only, and generally, no information on discharged patients. Due to traditionally installed rules in surgical techniques (gentle) and adherence to correct aseptic practice, disinfection and sterilization, preparative work and postoperative care for patients, the Incidence rate of SSI is stable < 3 - 5 % during the last 3 years. Paradoxically in February 2003 the SICU experienced a hospital outbreak with ESBL-producing *K. pneumoniae*, obviously related to the new non-trained personnel.

Microbiological surveillance data (Table 1) shows that *S. aureus* and *P. aeruginosa* are the most frequent nosocomial isolates from SSI. While methicillin-resistant staphylococci (MRSA) do not exceed 12 - 15 % last 3 years, the relative rate of multiresistant (ceftazidime-, aminoglycoside-, ciprofloxacin-, and 13 - 30 % carbapenem-resistant) *P. aeruginosa* increases.

Antibiotic consumption is high, particularly in SICU (Table 2), where the number of patients with severe infections, comorbidity and infections due to antibiotic resistant strains increases.

In order to better control SSI and appropriate usage of antibiotics, Hospital administration gave priority to the issues associated with the Control of Infection and Rational Antibiotic Policy. Futuremore a special Form for Antibiotic Prophylaxis and Therapy in Surgery was introduced - to be used for audit of antibiotic prescriptions. Data on Antibiotic prophylaxis in Surgery from the first trimester 2003 were completed, discussed and analyzed by anesthetists, sur-

Table. 1. Microbiological surveillance of etiologic structure of infections in Surgery and important antibiotic resistance

Microorganisms	2003	2004
Gram-positive organisms	35%	42%
S. aureus	7%	12%
MRSA	7%	24%
Aerobic Gram-negative	34%	29%
Enterobacteriaceae with ESBL	17%	12%
P. aeruginosa and other GNNF	25%	23%
P. aeruginosa poly-resistant	10%	26%
Candida spp	5%	5%
Anaerobes	3%	3%

Legend: GNNF - Gram-negative non-fermenters

Table. 2. Antibiotic consumption in Surgical Intensive Care Unit

Year	Number DDD/ 100 bed-day
2001	169.8
2002	274.7
2003	164.0

Table 3. Audit of Antibiotic prophylaxis in Surgery, the first trimester 2003

Gastro-duodenal procedures with a high risk	**International guidelines**
Augmentin 3 x 1.2 g (4)	Cefazolin/Cefoxitin (x 1)
Ampicillin/Sulbactam 3 x 3 g (1)	
Ceftriaxone 2 x 1 g (2)	
Augmentin 3 x 1 g + Amikacin x 1 g (1)	
Cefoperazone 3 x 2 g + Amikacin x 1 g (1)	
Cefepime 2 x 1 g + Metronidazole 2 x 1 g (1)	
Bile tract procedures with high risk	
Augmentin 3 x 1.2 g (3)	Cefazolin/Cefoxitin (x 1)
Cefazolin 3 x 2 g (3)	
Ceftriaxone 2 x 1 g (4)	
Cefazolin 3 x 2 g + Amikacin x 1 g (1)	
Ceftriaxone 2 x 2 g + Metronidazole 2 x 0.5 g (2)	
Ceftriaxone 2 x 1 g + Amikacin x 1 g (1)	
Colo-rectal procedures	(previous day)
Augmentin 3 x 1.2 g (3)	PO neomycin + erythromycin
Augmentin 3 x 1.2 g + Metronidazole 2 x 0.5 g (2)	IV cefoxitin/cefotetan or
Cefazolin 3 x 2 g + Metronidazole 2 x 0.5 g (5)	IV cafazolin + metronidazole
Ceftriaxone 2 x 1 g + Metronidazole 3 x 2 g (1)	
Cefazolin 3 x 2 g + Amikacin x 1 g + Metronidazole 3 x 0.5 g (2)	
Augmentin 3 x 1.2 g + Amikacin x 1 g + Metronidazole 3 x 0.5 g (5)	
Ceftriaxone 2 x 1 g + Amikacin x 1 g + Metronidazole 3 x 0.5 g (1)	
Urological procedures with high risk	
Pefloxacin 2 x 0.4 g (1)	ciprofloxacin/co-trimoxazole
Augmentin 3 x 1.2 (1)	(or according to antibiogramme)
Ampicillin/Sulbactam 3 x 3 g (1)	
Cephalothin 3 x 2 g (1)	
Amikacin 1 x 1 g (7)	
Cefoperazone 2 x 1 g (1)	
Ceftriaxone 2 x 1 g (3)	
Cefazolin 3 x 2 g + Amikacin 1 x 1 g (1)	
Ceftriaxone 2 x 1 g +Amikacin 1 x 1 g (1)	
Ampicillin/Sulbactam 3 x 3 g + Cefepime 2 x 1 g + Metronidazole 2 x 1 g (1)	
Cefepime 3 x 1 g + Amikacin 1 x 1 g + Metronidazole 2 x 0.5 g (2)	

geons and microbiologists (Table 3). Typical examples of disagreement with published world Guidelines represent:
* Among Gastro-duodenal procedures (high risk) - 10 operations:
in 3 of them 3rd, 4th generation cephalosporins have been used: ceftriaxone, cefoperazone, cefepime; in 2 - amikacin was used.
* Among Bile tract procedures (high risk) - 14 operations:
in 3 of them ceftriaxonehas been used, in 2 - amikacin
* Among Colo-rectal procedures - 19 operations:
in 2 of them used ceftriaxone
* Among Urology procedures (adenectomia, cystectomia, nephrectomia) - 20 operations:
in 3 of them have been used 3rd gen cephalosporins, in 2 - cefepime, in 3 - amikacin.
Comment: too broad-spectrum antibiotics were used generally in therapy; in 46 % of the examples - longer duration of prophylaxis has been registered.
After this unfavorable analysis from the audit, a multi-disciplinary working group elaborated specific to our institute Antibiotic prophylaxis policy in Surgery, which has been widely discussed before approval.

DISCUSSION

Although the prophylaxis of SSI in the published Guidelines (Antibiotic prophylaxis and Infection control) seems well defined and easy to be established, the practice is more complex (7, 10-15). Most of patients have additional risk factors that should be considered in the particular case. Anesthetists and surgeons should actively participate both in writing and implementation of the institutional prophylaxis policy (14).
One of the most important issues in prophylaxis of SSI institutionally is the support by hospital administration.
In conclusion,
1. Guidelines for Antibiotic prophylaxis in Surgery should be adapted to the particular hospital by its own physicians. Although we have our own rules now, more is expected as it concerns the doctors compliance.
2. Prevention of Surgical Site Infections, incl. Control of Infection and Rational Antibiotic policy became priority items in our institute: the proves are: the renovation of Surgical departments the re-enforcement/creation of Infection control- and Antibiotic policy Committees, as well as the introduction of Form for antibiotic prophylaxis and therapy in Surgery; the audit of antibiotic usage and nosocomial infections. They are considered important necessary step in Phase 1 of our Project, aimed at prevention of Surgical Site infections.

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INVESTIGATION ON THE IMMUNE STATUS OF THE POPULATION AGAINST WHOOPING COUGH DURING THE PERIOD 2001 - 2004

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SUMMARY

The specific immunoprophylaxis of humans with pertussis, as a component of combine bacterial vaccine leads to production of specific antibodies that is indicator of the whooping cough prevention. For evolution of immunization procedures and the vaccine itself, antibody levels against pertussis are useful to show the immune status of the population. An enzyme-linked immunosorbent assay was used for measuring immunoglobolin G pertussis antibodies in human sera. The assay was done in plastic plates coated with inactivated bacterial cells. For investigation were tested 3 711 human sera in different age groups. The comparison of different groups of age showed that the best protection against whooping cough have in people younger than 25 years old. In these groups of age the sera without antibodies against pertussis are 7% and 11% of patients have titer of antibody more than 1:321, which titer is used as a criteria of disease. Aging of people leads to reduction of the percent of protected persons and to increasing the percent of non-protected people. Despite this, the percent of protected population in-groups of age between 16 to 55 years old is better than in some European countries and in the USA. The patients over 56 years old have low levels of antibodies against pertussis because the vaccination process in Bulgaria is began since 1958 and in about 55% lacked protection against whooping cough. The annual differences of the rate of protection levels during the investigation period were not observed. Present results indicate a good protection against pertussis in Bulgaria. This fact is a result of specific immunoprophylaxis by pertussis vaccine, as a component of combined bacterial vaccine, produced by BB - NCIPD, Ltd.

Key words: pertussis, whooping cough, ELISA, immune status.

Pertussis (whooping cough) is a respiratory infection caused by Bordetella pertussis strains. The disease has been effectively controlled by vaccine prophylaxis (6, 10). Most currently marketed pertussis vaccine is inactivated whole cell preparations, administrated in combination with diphtheria and tetanus toxoids (9).

Laboratory verification of whooping cough, which is almost exclusively based on isolation of the bacterial strain, is associated with several problems (8, 14). Therefore attempts have been made to develop serological assays (1, 6, 12). However, the agglutination - type assay to study to assess the immune response to pertussis vaccine and Bordetella pertussis infection in human serum is not practical for seroepidemiological and diagnostic purposes, regardless of its sensitivity and reproductivity (4, 11). Direct immunofluorescent antibody staining of nosopharyngeal secretions has been used for diagnosing whooping cough, but this test lacks specificity, and false - positive results of up to 40% have been described (3). We have recently developed an enzyme - linked immunosorbent assay (ELISA) that measures the level of antibody response against pertussis by detection of Ig G antibody (8, 14). The high sensitivity and specificity of the ELISA allowed for antibody determination in small amounts of serum (1).

Knowledge of the immune status of population against pertussis has big practicable implication (8, 10, 12). It may assist in checking the efficacy of immunization schedules adopted and the persistence of immunity. Seroepidemiological investigations, performed in various European countries (7) and, in particular, in Sweden (11) and Italy, have demonstrated that in a high percentage of the population, even in the younger age groups, the pertussis antibody titer is below the level considered to confer protection against pertussis. Similar data have also been reported for the USA (3, 15). Therefore was necessary to make a screening for level of the protection against pertussis of the population in Bulgaria.

In this paper we present our results of estimation of the immune status of the population by ELISA method for immunoglobolin G antibodies against whooping cough in human sera during the four years investigation period.

MATERIALS AND METHODS

Human sera.

The epidemiological study on pertussis immunity was carried out on 3 711 subjects, selected by Hygiene Epidemiological Inspection, Sofia, Bulgaria. Sera separated from clotted blood were stored at - 20°C in small vials and were tested for pertussis antibodies using ELISA method. According to person's age serum samples were divided in seven groups - children between 0 to 7 years old, age group between 8 to 15 years old, people between 16 to 25 years old, adults from 26 to 35 years old, adults from 36 to 45 years old, adults from 46 to 55 years old and persons over 56 years. The number of sera are performed on Table No. 1

The obtained results were classified depend of the titer of antibody in following subgroups: subgroup of sera with the level of antibody up to 1:80 - group of non protective people; subgroup with level of antibodies against pertussis between 1:81 to 1:160 - sera with basic immunity, subgroup with level of antibodies against pertussis between 1:161 - 1:320 - sera with full protection and subgroup with level of antibodies against pertussis more than 1:321, human sera with titer used as a criteria for illness or past disease (5).

ELISA (3).

The BA test (13) is used for preparation of serum standard for detection of the titers by ELISA. This was the first method developed to measure pertussis antibody and it is still the most frequently used method. Bordetella pertussis suspension (serotype 1, 2 and 3), (batch 36, BB - NCIPD, Ltd. - Sofia, Bulgaria), dued and inactivated with 0.01% thiomersal was used in BA test. Diluting the stock suspensions to opacity 10 OUs made working dilutions. Sera samples were prepared in following concentrations: 1:10; 1:20; 1:40; 1:80; 1:160; 1:320; 1:640; 1:1280; 1: 2560. The level of antibody is measured after 24 hours incubation at 37°C and following incubation for 24 hours at room temperature. The last dilution of human sera with agglutination is a titer of antibody against pertussis.Polystyrene plates with 96 wells (Nunc immunoplates, Denmark) were coated with bacterial cells in working dilutions 10 OUs (batch 39, BB - NCIPD, Ltd. - Sofia, Bulgaria). To prepare the plates the bacterial cells were diluted in chloroform: ethanol (1:10) (4). 100 (l volumes were added to the wells and left 2 hours at 370 C and overnight at room temperature. The following morning the plates were emptied and drained over filter paper. After being washed three times with PBST (Phosphate Buffered Saline pH 7.2 containing 0.05% Tween 20), 100 (l volumes blocking buffer - PBS containing 1% BSA (Bovine Serum

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ABBREVIATIONS USED IN THIS PAPER: PBS - phosphate buffered saline; OPD - ortophenilenediamine.

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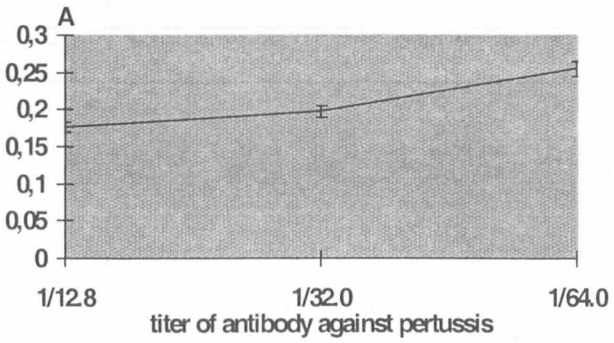
Table 1. Number of tested sera in different age groups.

Group of age	Number
Children between 0 to 7 years old	279
People between 8 to 15 years old	262
People between 16 - 25 years old	1 057
Adults from 26 to 35 years old	1 214
Adults from 36 to 45 years old	359
Adults from 46 to 55 years old	316
Persons over 56 years.	224
Total	3 711

Figure 1: ELISA: Standard curve.



Albumin) were added to the wells and incubated at 37°C for 1 hour.

100 µl volumes of sera dilutions (1:50) in PBST were added to the wells of sensitized plates, incubated at 37°C for 2 hours and washed three times in PBST. 100 µl volumes of peroxides anti - human immunoglobolin G (National Centre of Infectious and Parasitic Diseases - Sofia, Bulgaria) diluted in PBST (1:500) was added to each well, followed by incubation at 37°C for 60 minutes and washing three times of the plates. The plates were than left at room temperature with 100 µl volumes of a chromogen solution (10 mg of ortophenilenodiamine and 10 µl of 30% hydrogen peroxidase in 50 ml citrate - phosphate buffer pH 5.0). The reaction was stopped after 30 minutes with 100 µl of 1 N H_2SO_4. The plates were directly read at 405 nm in a MicroELISA Minireader Photometer (Bio-Tek) in optical densities. Standard curve with five points according to measured optical densities of used control serum samples, standartizated by BA method, was prepared each time (Fig. No. 1).

RESULTS AND DISCUSSION

We have tested 3 711 human sera by ELISA for estimation of pertussis antibodies and the results are presented on Table No. 2, 3, 4, 5.

The tested sera, of group of children till seven years of age, showed that between 51% to 53% of persons have full protection against whooping cough and in 43% to 45% of children the obtained titers showed basic immunity. In this group was not found subgroups of non-protected people. The titer of antibody against pertussis is more than 1:321 in 4% of human sera. All of sera have good level of protection against whooping cough and non-protected patients were not found.

The results of ELISA on human sera of age between 8 to 15 years old demonstrated that between 42% to 44% of persons have full protection, 47% to 50% have basic immunity and the antibodyьs level against pertussis in 4% to 5% of human sera is high than 1:321. In this group was found 4% to 5% non-protected people.

The patients between 16 to 25 years old have a good protection against pertussis. The titers obtained after assay showed that between 81% to 83% of human sera had titer of antigen defended a basic immunity. From 12% to 14% of the tested sera on this group have full protection and in 2% - 3% of the people the titer of antibody is on the levels, which used as a criteria of passed disease. Only 2% - 3% of sera are non - protective.

The analyses of immune status of the population 25 to 55 years old showed decrease of the titers of antibody against whooping cough compared with the younger groups. The sera with titer of antibodies from 1:81 to 1:160 in group of age between 26 to 35 are from 30% to 34% , in group of age between 36 to 45 are 54% - 60%, and in group of age between 46 to 55 are between 56% to 68%. The human sera with titer from 1:161 to 1:320 in group of age between 26 to 35 are 40% - 41%, in group of age between 36 to 45 are 19% - 25% and in group of age between 46 to 55 are 17% - 22%. The non-protected sera in group of age between 26 to 35 are 20% - 22%, in group of age between 36 to 45 are 17% - 18% and in group of age between 46 to 55 are 14% - 15%.

The persons over 56 years old have very low titer of antibodies against pertussis. The obtained results showed that only 43% - 45% of sera have protection against pertussis and 53% - 55% are non-protected.

The ELISA involves the blinding of bacterial cells to polystyrene tubes. Results of the direct ELISA test are highly reproducible (2). It is believed that a pertussis antibody level of 1:81 provides protection against disease. The titers of antibody from 1:161 to 1:320 showed full protection of people against whooping cough and titers over 1:321 are used as a criteria of disease or used as a criteria of passed disease (5). The results of estimation of immune status of population in Bulgaria in different groups of age were based on the same criteria.

Summarized results of serological studies for four years, presented on Table No.6, showed that the best protection against pertussis had seen in people up to 25 years old (more than 90%). The increase of age in people leads to reduction of the percent of persons with full protection and increase of sera with basic immunity and non - protected people. 55 % of the tested sera in age group over 56 years old are non- protected because the vaccination program in Bulgaria started since 1958. The prevalence of pertussis antibody in various age groups in the general population

Table 2. Estimation of the immune status of population in 2001.

Group of age	2001				
	Number of human sera	% full protection 1:161 - 1:320	% basic immunity 1:81 - 1:160	% non - protected less than 1:80	% of people with titer more than 1:321
Children between 0 to 7 years old	18	53	43	0	4
People between 8 to 15 years old	8	43	47	5	5
People between 16 - 25 years old	303	12	83	3	2
Adults from 26 to 35 years old	271	41	30	22	7
Adults from 36 to 45 years old	100	19	60	18	3
Adults from 46 to 55 years old	63	17	68	15	0
Persons over 56 years	43	0	43	55	2

Table 3. Estimation of the immune status of population in 2002.

Group of age	2002				
	Number of human sera	% full protection 1:161 - 1:320	% basic immunity 1:81 - 1:160	% non - protected less than 1:80	% of people with titer more than 1:321
Children between 0 to 7 years old	19	51	45	0	4
People between 8 to 15 years old	16	44	46	5	5
People between 16 - 25 years old	272	14	81	2	3
Adults from 26 to 35 years old	235	40	31	22	7
Adults from 36 to 45 years old	96	20	59	17	4
Adults from 46 to 55 years old	78	17	68	15	0
Persons over 56 years	56	0	43	55	2

Table 4. Estimation of the immune status of population in 2003.

Group of age	2003				
	Number of human sera	% full protection 1:161 - 1:320	% basic immunity 1:81 - 1:160	% non - protected less than 1:80	% of people with titer more than 1:321
Children between 0 to 7 years old	200	51	45	0	4
People between 8 to 15 years old	178	42	49	5	4
People between 16 - 25 years old	400	14	81	2	3
Adults from 26 to 35 years old	600	40	31	22	7
Adults from 36 to 45 years old	200	20	59	17	4
Adults from 46 to 55 years old	193	17	58	15	10
Persons over 56 years	69	0	43	55	2

Table 5. Estimation of the immune status of population in 2004.

Group of age	2004				
	Number of human sera	% full protection 1:161 - 1:320	% basic immunity 1:81 - 1:160	% non - protected less than 1:80	% of people with titer more than 1:321
Children between 0 to 7 years old	42	53	44	0	3
People between 8 to 15 years old	68	44	50	4	4
People between 16 - 25 years old	82	13	83	2	2
Adults from 26 to 35 years old	108	40	34	20	6
Adults from 36 to 45 years old	63	25	54	17	5
Adults from 46 to 55 years old	35	22	56	14	8
Persons over 56 years	56	1	45	53	1

Table 6. Estimation of the immune status of population during the period 2001-2004.

Group of age	2001 - 2004				
	Number of human sera	% full protection 1:161 - 1:320	% basic immunity 1:81 - 1:160	% non - protected less than 1:80	% of people with titer more than 1:321
Children between 0 to 7 years old	279	52	44	0	4
People between 8 to 15 years old	262	43	48	5	4
People between 16 - 25 years old	1 057	13	82	2	3
Adults from 26 to 35 years old	1 214	40	32	21	7
Adults from 36 to 45 years old	359	22	57	17	4
Adults from 46 to 55 years old	316	18	63	14	5
Persons over 56 years	224	0	43	55	2

depends on the status of immunization against pertussis in childhood.

Previous pertussis vaccinations of patients with whooping cough may interfere with the antibody response to the natural disease (13). Granstorm et al. (7, 8) found that the antibody response in unvaccinated people different in their vaccinated counterparts: unvaccinated children had an early increase in Ig M titers and late Ig G response (more than 1:131), whereas most vaccinated children and adults had a secondary type response with an early increase in Ig A and the level of Ig G antibody is until 1:320.

Populations in other countries that have also been studied in this way appear to be somewhat heterogeneous on the basis of these criteria (2). However, according to our opinion, in most cases the population can be considered comparable and from this comparison, the epidemiological situation observed in Bulgaria appears more favorable than that of some European countries and of the USA (2, 3, 7). In Sweden (1), for instance, high proportion of children had experienced pertussis infection by age 10, in Czechoslovakia the percent of sero- positively decreased from 81% in persons 15 to 19 years old to 16% in persons 30 to 34 years (15) and in Poland the children to one year the proportion with titer 1:40 or higher was 60%, while the proportion with a titer 1:160 or higher was 29%. Only 7% of person aged 15 years in Poland had a titer of 1:160 or higher (5).

The obtained results indicate a good protection against pertussis in Bulgaria. This is a result of specific immunoprophylaxis with pertussis vaccine, as a component of combined bacterial vaccines following the shame recommended by the Bulgarian immunization program.

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DIAGNOSTICS OF THE FIRST SUSPECT HUMAN CASES OF AVIAN INFLUENZA A/H5N1/ VIRUS IN BULGARIA

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SUMMARY

The first diagnostic investigations have been made, demonstrating avian flu A/H5N1/ virus etiological role. For the period January-March 2006 twenty six suspect patients, having been in close contact with ill or dead birds, and with a subsequent respiratory illness, were tested. Specific A/H5N1/ assays were applied /GeNet Bio rapid antigen detection test and Sacace RT-PCR kit/. Viral isolation was performed using MDCK cell lines and chicken embryos. Avian flu A/H5N1/ virus as a causative agent of respiratory disease was neither isolated, nor detected in any one of the tested patients. Subtype A/H1N1/ human influenza virus, identifiable by HIT and RT-PCR, was isolated in three of the patients tested.

Key words: avian influenza A/H5N1/ virus, diagnostics

The avian influenza (AI), caused by certain highly pathogenic subtypes of influenza A virus in animal populations, and particularly chickens, poses a continual health risk for the human public on a global scale. Direct human infection by avian influenza A (H5N1) virus was first recognized during the 1997 outbreak in Hong Kong. Till April 2006, the cumulative number of the confirmed human cases of avian influenza A/(H5N1) attained 193 cases, of which 109 of lethal outcome. (WHO. Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) 11.04.06).

Because of Bulgaria's crossroad location regarding the wild birds main migratory routes, a real danger of disease transmission exists among the avian population in our country. In the beginning of 2006, A (H5N1) has caused an infection among the poultry in the neighbouring countries /Turkey, Romania, Ukraine/. The virus has been isolated from wild swans in many European countries, Bulgaria including. All these circumstances favor the appearance of the first suspect human cases of avian influenza A(H5N1) in the country. The latter necessitates effective diagnosis not only of human influenza infections, but of avian influenza infections as well. The laboratory identification of influenza virus infections is commonly accomplished by antigen detection, isolation in cell culture, or detection of influenza-specific RNA by (the) highly sensitive and specific reverse transcriptase-polymerase chain reaction /RT-PCR/ (WHO.Recommended laboratory tests to identify avian influenza A virus in specimens from humans. June 2005)

The aim of the survey is the investigation of the first suspect human cases of avian influenza A/H5N1/ virus, using viral isolation and rapid tests of antigen and genome detection.

MATERIALS AND METHODS

Clinical specimens: nasal and throat swabs from twenty six patients with influenza like diseases were examined. All the patients were in close contact with ill or dead birds or travellers in countries with registered avain flu cases. Post-mortem lung, tracheal tissue and bronchoalveolar lavage from two patients were also tested. All the samples were collected during the period January -March 2006. Patients' data /sex, age, source of hypothetical contamination / is shown on Table 1.

Viral isolation was performed by one passage in MDCK and subsequently two passages in chicken embryos. The isolated strains were identified by HIT. The preliminary screening of the samples was made by rapid H5 antigen detection immunosorbent test GeNet Bio.

RT-PCR for detection of viral RNA was performed with the initial samples. RNA was extracted by using monophasic solution of phenol and guanidinium isothiocyanate-Trizol LS (Invitrogen life technologies, USA) an improvement of the single-step RNA isolation method as developed by Chomezynski and Sacchi.

RT-PCR avian Influenza A Virus (H5, H7) Screening and Typing kit (Sacace, Italy) was used for detection of probable A/H5N1/ samples. Conventional RT-PCR for human influenza viruses by using One-Step Ready-to-Go RT-PCR Beads (Amersham Biosciences, U.K) kit for simultaneous cDNA synthesis and PCR reaction was applied. HA and NA oligonucleotide primers were selected from highly conserved regions of the hemagglutinin and neuraminidase gene segments of the influenza viruses using two specific primer pairs for subtype A (H1N1) and A (H3N2). (Hadzhiolova T., Contemporary methods for influenza virus diagnosis and genetic characterization of influenza viruses, PhD thesis -February 2006, Sofia).

The obtained PCR products (amplicons) were analyzed using 2% agarose gel electrophoresis (100 V for 1 h 30 min.) and their sizes were compared with a DNA molecular weight marker ((X-174 or 100 bp) (Invitrogen life technologies, USA). Positive H5 RNA or standard influenza strains, as well as negative (ddH2O) controls were included the experiments. The results were read by MiniBis Pro DNR (Bio-imaging Systems, Israel).

In March 2006 we have examined five additional samples received in connection with International Control for human and avian influenza by the Institute for standardization and documentation of medical laboratories (INSTAND). The samples were screened by Directigen Flu A+B (Becton&Dickinson, USA) for detection of viral NP and differentiation of type A or B influenza viruses. Specific H5 antigen detection kit (GeNet Bio, Korea) and RT-PCR avian Influenza A Virus (H5, H7) Screening and Typing kit (Sacace, Italy) were used for detection of A/H5N1/ samples.

RESULTS AND DISCUSSION

Wnen examining the first suspect A/H5N1/ patients in Bulgaria, the diagnostic scheme stages as developed by the National Influenza Laboratory for human influenza viruses, have been followed. By reason of the disease severity and the necessity of rapid diagnostic response, the scheme was realized as follows:

MDCK cell lines were used for inoculation of the clinical materials immediately following their receipt in the laboratory. Chicken embryos for minimum two consecutive passages have been continuously supplied. Viral isolation has been carried out for 5-7 days.

Instant screening of the initial samples by rapid antigen detection tests and RT-PCR for typing and subtyping of viral RNA.

Despite the usage of two laboratory models, and the performance of three passages, avian influenza viruses were not isolated from any one of the 26 suspect patient's samples. The same negative result for A/H5N1/ was confirmed by the preliminary screening of the initial samples using GeNet Bio rapid antigen detection test, as well as

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ABBREVIATIONS USED IN THIS PAPER: AI - Avian Influenza

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Table 1. Suspect A/H5N1/ patients investigated in the laboratory of Influenza for the period January-March 2006

Patient №	sex	age	Town, village/ probable source of contamination
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2	f	27	Pleven/ contact with ill bird
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4	m	21	Chinese citizen
5	m	39	Varna/dead swan
6	m	45	Shabla/dead swan
7	f	11	Shabla/dead swan
8*#	f	27	Plovdiv/ fast food staff
9#	m	31	Haskovo/dead bird
10	m	16	Shabla/dead swan
11	m	38	Shabla/dead swan
12	f	36	Shabla/dead swan
13	m	48	Turkish citizen
14	f	81	Silistra /dead swan
15	m	58	Silistra /dead swan
16	m	54	Silistra /dead swan
17	f	54	Silistra /dead swan
18	f	38	Shumen/ veterinarian, dead swan
19	m	60	Silistra /dead swan
20	f	22	Silistra /dead swan
21	m	45	Plovdiv/ ill bird
22*	m	49	Vidin / ill bird
23#	m	9	Sliven/dead hen
24	m	36	Dobrich /ill dove
25	m	47	Haskovo/ traveler to China
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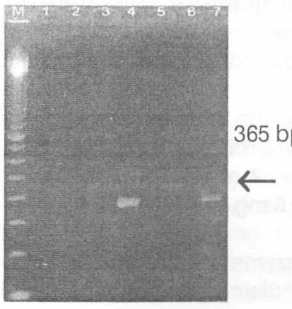
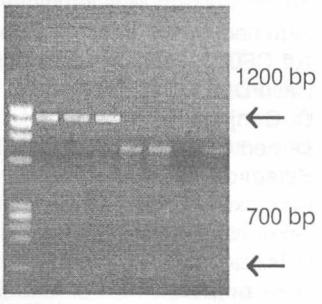
Figure 1. RT-PCR for detection of A/H5N1/ avian influenza virus



Legend: Fig. № 1

M	DNA100 bp marker
Line 4	(+) sample for avian influenza virus A/H5/
Line 5	(-)control
Line 7	(+)control

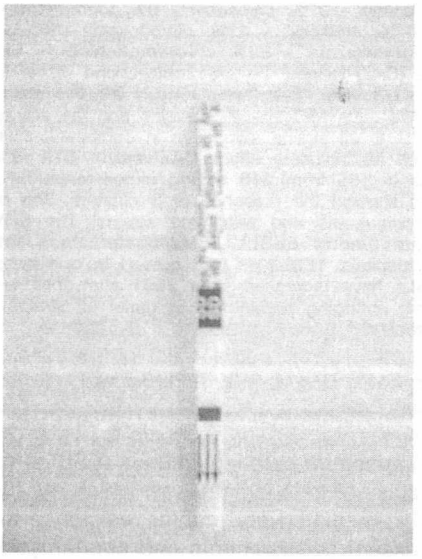
Figure 2. RT-PCR for detection of A/H1N1/ human influenza virus



Legend: Fig. № 2

M	Φx-174 marker
Lines 1-3	(+) samples for human influenza virus A/H1/
Line 6	(-)control
Lines 4,5 и 7	(+)samples for human influenza virus A/N1/

Fig.№3. GeNet Bio rapid A/H5/ test



RT-PCR (Sacace, Italy) kit. The sensitivity and specificity of the molecular kit was demonstrated when examining the samples by the International Control, one of the specimens being determined as positive for A/H5/ RNA (Fig. 1).

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The results presented here in summarize the preliminary data regarding avian influenza A/H5N1/ virusњ diagnostics in humans. We consider RT-PCR (Sacace, Italy) kit adequately effective as regards screening and genotyping of probable A/H5N1/ samples, in spite of the lack of H5 positive clinical sample to the present moment.

The negative result obtained by GeNet Bio (Fig. 3) for the sample received by INSTAND and tested positive by RT-PCR makes us doubt the rapid test sensitivity for avian influenza virus antigen detection in clinical samples.

The investigation of the first suspect A/H5N1/ avian influenza virus patients allowed us to acquire the useful skills when working with highly pathogenic infectious agents, and to gain practical experience when applying the necessary diagnostic methods for viral isolation and influenza H5 antigens and genome detection for extremely short period of time.

REFERENCES:

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2. WHO. Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1). 11.04.06.
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DIAGNOSTICS OF THE FIRST SUSPECT HUMAN CASES OF AVIAN INFLUENZA A/H5N1/ VIRUS IN BULGARIA

T. Hadzhiolova, S. Pavlova, R. Kotseva

SUMMARY

The first diagnostic investigations have been made, demonstrating avian flu A/H5N1/ virus etiological role. For the period January-March 2006 twenty six suspect patients, having been in close contact with ill or dead birds, and with a subsequent respiratory illness, were tested. Specific A/H5N1/ assays were applied /GeNet Bio rapid antigen detection test and Sacace RT-PCR kit/. Viral isolation was performed using MDCK cell lines and chicken embryos. Avian flu A/H5N1/ virus as a causative agent of respiratory disease was neither isolated, nor detected in any one of the tested patients. Subtype A/H1N1/ human influenza virus, identifiable by HIT and RT-PCR, was isolated in three of the patients tested.

Key words: avian influenza A/H5N1/ virus, diagnostics

The avian influenza (AI), caused by certain highly pathogenic subtypes of influenza A virus in animal populations, and particularly chickens, poses a continual health risk for the human public on a global scale. Direct human infection by avian influenza A (H5N1) virus was first recognized during the 1997 outbreak in Hong Kong. Till April 2006, the cumulative number of the confirmed human cases of avian influenza A/(H5N1) attained 193 cases, of which 109 of lethal outcome. (WHO. Cumulative Number of Confirmed Human Cases of Avian Influenza A/(H5N1) 11.04.06).

Because of Bulgaria's crossroad location regarding the wild birds main migratory routes, a real danger of disease transmission exists among the avian population in our country. In the beginning of 2006, A (H5N1) has caused an infection among the poultry in the neighbouring countries /Turkey, Romania, Ukraine/. The virus has been isolated from wild swans in many European countries, Bulgaria including. All these circumstances favor the appearance of the first suspect human cases of avian influenza A(H5N1) in the country. The latter necessitates effective diagnosis not only of human influenza infections, but of avian influenza infections as well. The laboratory identification of influenza virus infections is commonly accomplished by antigen detection, isolation in cell culture, or detection of influenza-specific RNA by (the) highly sensitive and specific reverse transcriptase-polymerase chain reaction /RT-PCR/ (WHO.Recommended laboratory tests to identify avian influenza A virus in specimens from humans. June 2005)

The aim of the survey is the investigation of the first suspect human cases of avian influenza A/H5N1/ virus, using viral isolation and rapid tests of antigen and genome detection.

MATERIALS AND METHODS

Clinical specimens: nasal and throat swabs from twenty six patients with influenza like diseases were examined. All the patients were in close contact with ill or dead birds or travellers in countries with registered avain flu cases. Post-mortem lung, tracheal tissue and bronchoalveolar lavage from two patients were also tested. All the samples were collected during the period January -March 2006. Patients' data /sex, age, source of hypothetical contamination / is shown on Table 1.

Viral isolation was performed by one passage in MDCK and subsequently two passages in chicken embryos. The isolated strains were identified by HIT. The preliminary screening of the samples was made by rapid H5 antigen detection immunosorbent test GeNet Bio.

RT-PCR for detection of viral RNA was performed with the initial samples. RNA was extracted by using monophasic solution of phenol and guanidinium isothiocyanate-Trizol LS (Invitrogen life technologies, USA) an improvement of the single-step RNA isolation method as developed by Chomezynski and Sacchi.

RT-PCR avian Influenza A Virus (H5, H7) Screening and Typing kit (Sacace, Italy) was used for detection of probable A/H5N1/ samples. Conventional RT-PCR for human influenza viruses by using One-Step Ready-to-Go RT-PCR Beads (Amersham Biosciences, U.K) kit for simultaneous cDNA synthesis and PCR reaction was applied. HA and NA oligonucleotide primers were selected from highly conserved regions of the hemagglutinin and neuraminidase gene segments of the influenza viruses using two specific primer pairs for subtype A (H1N1) and A (H3N2). (Hadzhiolova T., Contemporary methods for influenza virus diagnosis and genetic characterization of influenza viruses, PhD thesis -February 2006, Sofia).

The obtained PCR products (amplicons) were analyzed using 2% agarose gel electrophoresis (100 V for 1 h 30 min.) and their sizes were compared with a DNA molecular weight marker ((X-174 or 100 bp) (Invitrogen life technologies, USA). Positive H5 RNA or standard influenza strains, as well as negative (ddH2O) controls were included the experiments. The results were read by MiniBis Pro DNR (Bio-imaging Systems, Israel).

In March 2006 we have examined five additional samples received in connection with International Control for human and avian influenza by the Institute for standardization and documentation of medical laboratories (INSTAND). The samples were screened by Directigen Flu A+B (Becton&Dickinson, USA) for detection of viral NP and differentiation of type A or B influenza viruses. Specific H5 antigen detection kit (GeNet Bio, Korea) and RT-PCR avian Influenza A Virus (H5, H7) Screening and Typing kit (Sacace, Italy) were used for detection of A/H5N1/ samples.

RESULTS AND DISCUSSION

Wnen examining the first suspect A/H5N1/ patients in Bulgaria, the diagnostic scheme stages as developed by the National Influenza Laboratory for human influenza viruses, have been followed. By reason of the disease severity and the necessity of rapid diagnostic response, the scheme was realized as follows:

MDCK cell lines were used for inoculation of the clinical materials immediately following their receipt in the laboratory. Chicken embryos for minimum two consecutive passages have been continuously supplied. Viral isolation has been carried out for 5-7 days.

Instant screening of the initial samples by rapid antigen detection tests and RT-PCR for typing and subtyping of viral RNA.

Despite the usage of two laboratory models, and the performance of three passages, avian influenza viruses were not isolated from any one of the 26 suspect patient's samples. The same negative result for A/H5N1/ was confirmed by the preliminary screening of the initial samples using GeNet Bio rapid antigen detection test, as well as

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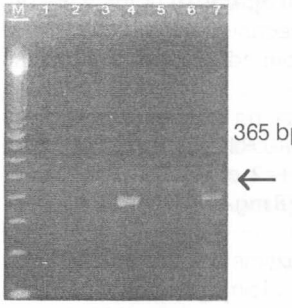

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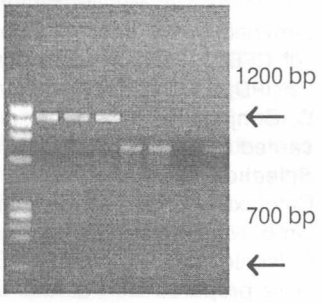

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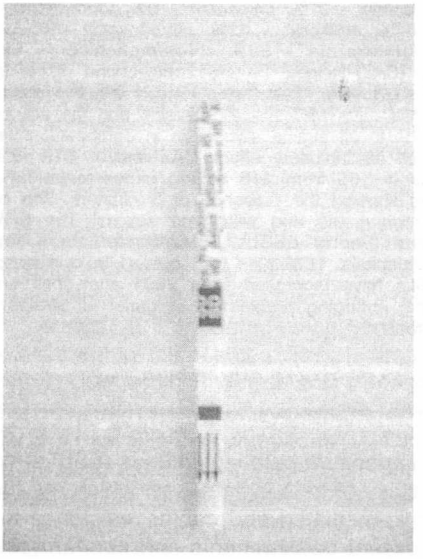

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EXTENDED SPECTRUM BETA-LACTAMASE (ESBL) PRODUCERS AMONG ENTEROBACTERIACAE FROM PATIENTS IN BULGARIAN HOSPITALS

R. Markovska[1], E. Keuleyan[1,2], M. Sredkova[3], D. Ivanova[4], B. Markova[5], K. Rachkova[6], E. Dragijeva[7], I. Haydouchka[8], T. Kostayanev[1], I. Mitov[1]

Departments of Microbiology: Medical Universities -Sofia[1],-Pleven[3], - Stara Zagora[6] -Plovdiv[8]; Medical Institute, Ministry of the Interior - Sofia[2]; "Queen Joanna" Univ. Hospital- Sofia[4]; Alexandrovska Hospital[5]; Pediatric University Hospital - Sofia[7]

SUMMARY

To preliminary characterize the main types of ESBLs among Bulgarian Enterobacteriaceae strains and to determine their rate of resistance. Methods: 458 Enterobacteriaceae (K.pneumoniae - 243, K. oxytoca - 9, E.coli - 156, C. freundii - 12, Enterobacter spp - 18, Proteus spp - 4, Serratia spp -15, Salmonella enterica Serotype Corvallis -1) strains identified as ESBL producers were collected from 8 medical centers in Sofia, Pleven, Stara Zagora and Plovdiv during 1996-2003. They were confirmed by phenotypic tests (DDS, NCCLS method). MICs were determined by an agar dilution technique (NCCLS, 2002). Conjugative plasmid transfer was performed, followed by an isoelectric focusing according to Mathew/Bauernfeind. The hydrolytic activity of the bands was proved by Bioassay (Bauernfeind).PCR experiments were used to confirm the type of ESBL. Results: The rate of resistance was: amoxicillin/clavulanate - 86%, ceftazidime - 85% , cefotaxime-95%, ceftriaxone - 94%, aztreonam - 74%, cefoxitin - 14%, ceftibuten - 19%, cefepime - 37%, imipenem - 0%, tobramycin - 95%, gentamicin - 84%, amikacin - 41%, ciprofloxacin -56%, tetracycline -86%, co-trimoxazole - 53% chloramphenicol - 58%. MIC of ceftazidime (CAZ) ranged 1 - >512 mg/L and of cefotaxime (CTX) - 2 - 512 mg/L The strains were divided into two main groups: the first one- $MIC_{CAZ} \geq MIC_{CTX}$ - 279 strains and the second: $MIC_{CAZ} < MIC_{CTX}$ -179. In all strains sulbactam in combination with CAZ and CTX showed an inhibitory effect. CAZ and/or CTX resistance was transferable in 185 from 216 mating experiments. IEF analysis of 247 strains showed the presence of 3 clusters. The pI data were from transconjugants and wild type strains. The SHV type was predominant among ESBLs Enterobacteriaceae producers in Bulgarian hospitals. TEM type was proved in one hospital. CTX-M types ESBLs have increased from 2001 after their first detection and become emerging problem in Bulgaria. All strains were highly polyresistant.

Key wards: ESBL, CTX-M, SHV, TEM, Bulgaria

Extended spectrum beta-lactamases (ESBLs) constitute a growing class of beta-lactamases, which are the most often plasmid-mediated and confer resistance to the beta-lactams except carbapenems and cephamycins [1]. The members of the family *Enterobacteriaceae* are among the most common pathogens causing urinary tract infections, nosocomial pneumonia, septicemia and intraabdminal infections. Production of ESBLs in this family has become one of the big problems for the antimicrobial therapy during the last years (1-6). First report for ESBL in Bulgaria was in 1992(2). The increasing use of cephalosporins of 3rd generation in Bulgarian hospitals supported the selection and dissemination of ESBL producing strains (1,6). Surveys in Bulgaria showed that ESBLs became emerging problem in our country and need more detailed investigation.

THE AIM of this work was:

1. To preliminary characterize the main types of ESBLs among Bulgarian Enterobacteriaceae strains by analytical isoelectric focusing.
2. To determine the rate of resistance of 458 ESBL producing strains.

MATERIALS AND METHODS

In total 458 strains (*K.pneumoniae* - 243, *K. oxytoca* - 9, *E.coli* - 156, *C. freundii* - 12, *Enterobacter spp* - 18, *Proteus spp* -4, *Serratia spp* -15, Salmonella enterica Serotype Corvallis -1) were collected from 8 medical centers in Sofia, Pleven Stara Zagora and Plovdiv during 1996-2003. They were derived from different specimens: blood and central catheter, broncho-tracheal secretions, urine, wound, drain, sputum, stomach sound, bile, feces and others.

A. Confirmation of ESBL production was by:

1. Double disk synergy (DDS) method by Jarlier et al with disks of 3rd generation cephalosporins and aztreonam.
2. NCCLS disk confirmatory method

B. Susceptibility testing was according to NCCLS 2002 guidelines with Mueller Hinton II agar (BD). MICs were determined by an agar dilution technique with inoculum of 10^4 CFU/spot. DDM was performed with antibiotic disks (NCIPD, Sofia and BBL, BD).

C. Conjugative plasmid transfer on a solid medium was carried out with E.coli K12: W3110 Rif lac (-), J62 Nx lac (-). Selection: Mc Conkey agar + 2 mg/L Ceftazidime or Cefotaxime 2 mg/L, or Cefoxitin 8 mg/L + 50 mg/L Rifampicin or Nalidixic acid 50 mg/L.

D. Isoelectric focusing (IEF): Enzyme extracts of the strains were prepared with ultrasonic disintegration of bacterial cultures. IEF was performed by a procedure of Mathew et al (3) modified by Bauernfeind (4) with polyacrylamid gel 30% containing Ampholyte pH 3-10.

E. Bioassay was performed by a procedure of Bauernfeind et al (4) to reveal the hydrolytic activity of the bands. The growth of the indicator strain identifies the position of which the chosen antibiotic has been inactivated.

F. Whole cell DNA preparations were used as template in specific PCRs to confirm genes coding for beta -lactamases of the TEM-, VEB-, SHV-, OXA-group I, II, III or CTX-M-groups.

RESULTS AND DISCUSSION

All tests for detection of ESBLs have shown a good agreement. In DDS test 308 from 458 strains were positive.

NCCLS confirmatory method was positive in all tested strains only for combination Ceftazidime and Ceftazidime + Clavulanic acid. For combination with Cefotaxime there were 26 from 409 *E. coli* and *K. pneumoniae* strains non-detected with this method.

The resistance to cephalosporins 3rd and 4th generation and aztreonam was not 100%, however in case of in vitro susceptibility of ESBL producers, their therapeutic use should be avoided, because of treatment failure. (5,6).

The strains showed high rate of resistance to TOB - 85%, GEN - 84% and TET - 86% (Fig 1). The susceptibility to SXT-47%, AMK -59%, CIP - 44% and CHL - 42% (Fig 1) also was decreased and became problematic. The rates of the resistance to these antimicrobials have shown that their use

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ABBREVIATIONS USED IN THIS PAPER: ESBL - extended spectrum beta-lactamases; PCR- polymerase chain reaction: CHL-chloramphenicol; SXT-co-trimoxazole; TET-tetracycline; CIP-ciprofloxacin; AMI-amikacin; GEN-gentamicin; TOB-tobramycin; IMP-imipenem; FEP-cefepime; BUT-ceftibuten; FOX-cefoxitin; AZT-aztreonam; CRO-ceftriaxone; CTX-cefotaxime; CAZ-ceftazidime; AUG-amoxicillin/clavulanic acid

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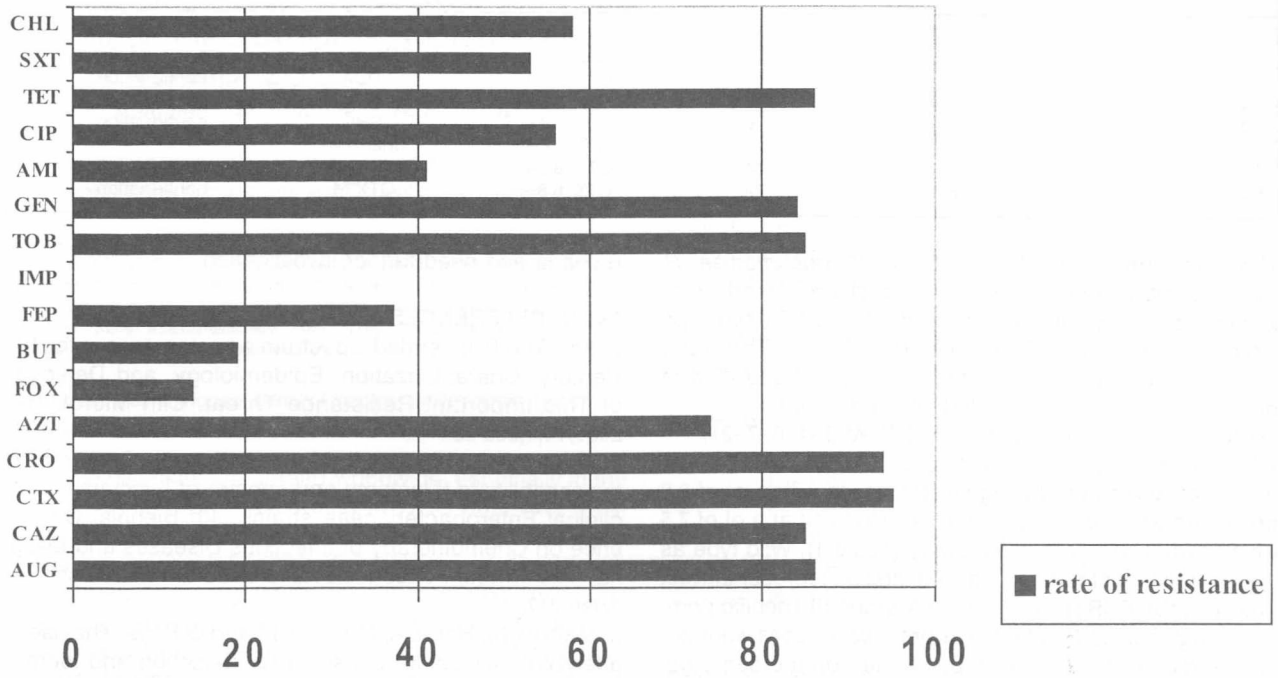
Figure 1. Rate of antimicrobial resistance in % among 458 ESBL producers in disk diffusion method



Abreviations: CHL-chloramphenicol; SXT-co-trimoxazole; TET-tetracycline; CIP-ciprofloxacin; AMI-amikacin; GEN-gentamicin; TOB-tobramycin; IMP-imipenem; FEP-cefepime; BUT-ceftibuten; FOX-cefoxitin; AZT-aztreonam; CRO-ceftriaxone; CTX-cefotaxime; CAZ-ceftazidime; AUG-amoxicillin/clavulanic acid

for the therapy needs of preliminary confirmation of the susceptibility.

The strains were 100% susceptible to IMP (with MIC < 0.5 mg/L). Carbapenems are recommended for serious infection as empirical therapy (5). The combination aminopenicillins and beta-lactamase inhibitors can be used in the cases of urinary tract infections and proven in vitro susceptibility (in high dosage).

MIC of CAZ ranged from 1 mg/L to >512 mg/L and that of CTX from 2 mg/L to 512 mg/L. For all strains sulbactam in combination with ceftazidime or cefotaxime showed an inhibitory effect.

Upon the susceptibility results the strains were divided into two main groups:

- zone of inhibition of CAZ < zone of inhibition of CTX - 279 strains, which suggested CAZ hydrolytic activity of the enzyme
- zone of inhibition of CAZ > zone of inhibition of CTX - 179 strains, which suggested CTX hydrolytic activity of the enzyme

Conjugative plasmid transfer of ESBLs was obtained in 185 of 216 mating. Ceftazidime or cefotaxime resistance determinants were located on a transmissible plasmid with or without other resistance markers.

Transconjugants were divided depending on the co-transferred markers:

- 9 GEN
- 49 TOB, GEN
- 17 TOB, AMI
- 12 TOB, GEN and TET
- 6 TOB, GEN and CHL
- 3 TOB, GEN and SXT
- 18 TOB, GEN, AMI and SXT
- 56 did not transfer other markers
- 3 TOB, GEN, TET and SXT
- 1 TOB, GEN, SXT and CHL
- 1 GEN, TET and SXT
- 1 GEN, AMI and SXT
- 3 TET, CHL
- 1 SXT, CHL
- 4 TET
- 1 CHL

Aminoglycoside resistance was most frequently co-transferred (120/185=64,9%) linked followed by resistance to SXT (15,1%), TET (12,4%) and CHL (6,5%).

IEF analysis of 247 strains showed, that the most strains contained 2 or 3 different beta-lactamases (Table 1). Mostly of them showed beta-lactamases focused at 5.4, which didn't hydrolyse cephalosporins 3rd generation - probably TEM-1. Other additional enzymes without hydrolytic activity were those, focusing at pI 7.0 or/and 7.6 - probably SHV broad-spectrum enzymes. The strains, investigated with isoelectric focusing can be divided into three main groups. The first group demonstrated beta-lactamases with CAZ hydrolytic activity and isoelectric point 7.6 or 8.2 suggesting SHV types. Ninety and eight strains, representing all included in the study Enterobacteriaceae genera showed beta-lactamases with pI 8.2 - Table 1. They hydrolysed CAZ and were visualized in both donor and transconjugant strains. We suggest SHV type ESBL, possibly SHV-5/-9/-12/-22/-45 (6) from which SHV-5 is ubiquitous (1). Such strains were found in all studied centers. Eight strains showed beta-lactamase focused at pI 7.6 with CAZ hydrolytic activity - Table 1. We can suggest SHV-2/-2a/-6/-7/-8/-13/-19/-20. PCR experiments confirmed production of SHV type beta-lactamases.

One K. pneumoniae strain showed a CTX hydrolysing beta-lactamase with a pI of 7.8 (Table 1). Its gene was transferred by conjugation. This strain gave negative PCR results with blaCTX-M, blaSHV and blaOXA 1, 2, 3 and blaVEB group specific primer sets. We can suggest new group beta-lactamase. This results show a need of more detailed investigation.

The second group: 48 strains (*K. pneumoniae*, *E.coli*, *C. freundii*) demonstrated beta-lactamases focusing at pI 6.3 (fig. 2), which were transferable in conjugation and hydrolysed CAZ. This suggests TEM type (the most probably TEM - 3 or TEM -16/-18/-22 /-89/-113) (6). The most strains were only from one center in Pleven.

One strain K. oxytoca presented one band at 5.4 with CAZ hydrolyzing activity. The mating experiment failed in this case. The strain gave negative result in PCR experiment with blaVEB and blaTEM primers. We can suggest probable OXY type beta-lactamase, which is chromosome mediated.

Table 1. Types of beta-lactamases according to IEF, Bioassay and PCR

pIs						Number of strains	Bioassay	Probable ESBL	PCR
5.4						1	CAZ 5.4+	TEM / PER	No confirmation
5.4	6.3	7.0/7.6				48	CAZ 6.3+	TEM	confirmation
5.4		7.0/7.6	8.2			98	CAZ 8.2+	SHV	confirmation
5.4		7.6				8	CTX 7.6+	SHV	confirmation
		7.8				1	CTX 7.8+	unknown	
5.4				8.6		14	CTX 8.6+	CTX-M	confirmation
5.4		7.5			8.8	78	CTX 8.8+	CTX-M	confirmation

The third group - 92 strains (*E.coli*, *K. pneumoniae*, *S. marcescens*, *E. cloacae*, *E. aerogenes*) presented beta-lactamases with pI between 8.4 and 8.8 and CTX hydrolyzing activity suggesting CTX-M type(1) - Table 1. They have been transferred in conjugation and hydrolysed CTX in bioassay.They were subdivided into two groups:

- With pI 8.4 - 14 strains (probable CTX-M-3,-4,-6,-7,-21)
- With pI 8.8 - 78 strains (probable CTX-M-15)

Almost all strains producing b-lactamases with a pI of 8.8 presented additional b-lactamases focussing at a pI of 7.5 which hydrolysed CTX in Bioassay (Table 1). Wild type as well as transconjugant strains, selected as representatives, gave positive PCR results with OXA-group III specific primers. The genes coding for these beta-lactamases were co-transferred, so they seem to be located on the same genetic element.

The strains, which were FOX resistant didn't transfer this marker. In this case we suggest changes or loss of outer membrane proteins.

CONCLUSIONS

1. The SHV type was predominant among ESBLs elaborated by 458 Enterobacteriaceae strains from Bulgarian hospitals. All studied centers harboured SHV type ESBL producing strains.
2. CTX-M types represented significant number of ESBL. They rapidly increased during the investigation period (first detection in 2001).
3. TEM type ESBL was found mainly in the strains from one medical center.
4. ESBLs producing strains became emerging problem in Bulgaria and need further investigation.

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ANTIHELMINTIC ACTIVITY OF NEW PIPERAZINE - CONTAINING BENZIMIDAZOLES

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SUMMARY

Three new synthetic analogues of the benzimidazole and piperazine hexahydrate have been developed. Experiments on white mice, infected with Syphacia obvelata show antihelminthic activity over 50% intens-efficacy /IE/. This result is promising and further investigation is necessary.

Key-words: helminthoses, benzimidazoles, antihelmintic actvity

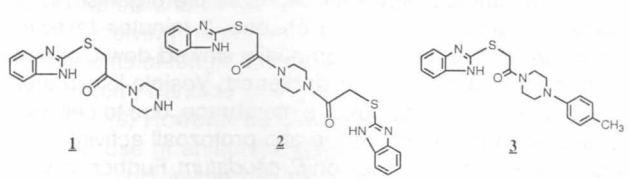
Parasitic diseases are spread worldwide, especially in the developing countries. According to WHO data over 3 billion of the world population is infected /3/. In our country, a number parasitic infections /ascaridosis, enterobiosis, hymenolepidosis, hydatidosis, trichinellosis etc./ still present a major public health concern. Treatment is often problematic, due to the lack of effective preparations. Continuous development and introduction of new therapeutic agents is therefore necessary /1, 2, 4/.

The aim of the present study is to test the antiparasitic activity of the synthesized by us new N-piperazine-benzimidazolyl-2-mercaptoacetamides. The preparations are tested on experimental animals, infected with the nematode Syphacia obvelata.

MATERIAL AND METHODS

Three N-piperazine-benzimidazole-2-mercapto acetamides are tested for antihelmintic activity (Fig.1).

Figure 1



Synthesis included incorporation in a single molecule of benzimidazole and piperazine nucleus structural faits of the commonly need agents Albendazole, Mebendazole, Diethylcarbamazine, Piperazin hexahydrat.

White mice, weghing 18-20 g were used in the experiment. Animals were assigued into 3 experimental groups /8 mice each/ and 3 control groups. All mice were infected with Syphacia obvelata.

The tested therapeutic agents were introdused intraoesophageally, using a thin metal probe, at a dose of 20 mg/kg b.w., once daily for 3 days. Water dimethylsulfoxide /DMSO/ has used as diluent /0.25 ml volume/.

Control group one received no treatment. Mice from group two were fed on the diluent DMSO only for three days. Control group 3 animals were given water solution of Piperazin hexahydrat /a widely used antiparasitic agents/ at a dose of 100 mg/kg for 3 days.

Tree days, following dicontinuation of treatment animal were killed and examined. A partial autopsy of the large intestines /permanent localisation of the mature S. obvelata parasites/ was performed. The number of adult parasites was determined, using a stereoscopic microscope.

Krotov's formula was need to establish the intens-efficacy /IE/ of the tested agents /2/.

$$IE = [(K-O):K].100,$$

IE - intens-efficacy

K - intensity of infection in the control /nontreated/ group

O - intensity of infection after treatment of the ex perimental group

Efficacy is assumed if IE > 50%.

RESULTS AND DISCUSSION

Intens-efficacy over 50% /IE 61% and IE 66% respectivlly/ was established for agents 2 and 3. Intens-efficacy of agent 1 was 11%. Treatment with Piperazin hexahydrate produsedd 61% intens-efficacy.

The intensity of infection was not reduced in mice, treated with DMSO and in those without treatment. The ahtihelmintic activity of agents 2 and 3, at a dose of 20 mg/kg was comparable to that of Piperazin hexahydrate, at a dose of 100 mg/kg.

Results of this study show that the therapeutic efficacy of agents 2 and 3 was over 50%. Further investigation of their antiparasitic activity is needed. The microscopic examination of the intestinal contents of the erxperimental animals revealed a large numbar of nematode larve. This fact give us the grounds to cxonsider that agents 2 and 3 are active against the adult parasites not agaonst the larvae.

Conclusion.

New synthetic analogues of the antiparasitic drugs Albendazole, Mebendazole, Diethylcarbamazine, Piperazin hexahydrat have been developed. Thair structure is proved using spectroscopy. Experiments show a therapeutic efficacy /IE/ of over 50% in agents 2 and 3. This efficacy considered promising and further investigation of the antiparasitic activity is necessary.

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ANTIPROTOZOAL EFFECT OF SOME NOVEL BIS-BENZIMIDAZOLES - AN EXPERIMENTAL STUDY

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SUMMARY

Novel bis-benzimidazole compounds with confirmed antiparasitic activity in helminthoses are synthesized. Experimental investigations on the antiprotozoal effect "in vitro" on Paramaecium caudatum are carried out. Chemotherapeutical tests "in vivo" on with mice infected with Lamblia muris are performed with the most effective bis-bensimidasoles. Some of the tested compounds have a well expressed antiprotozoal activity.

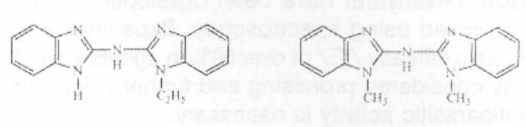
Key words: protozooses, experimental chemotherapy, bis-benzimidazoles

The benzimidazole preparations with antiparasitic activity are widely used in the treatment on helminthiases, caused by nematodes with various organic localization and less frequntly - by trematodes and cestodes.

In the past 10-15 years the antiprotozoal activity of different benzimidazoles has been investigated as well /2/. Experimentaly, the effect of Mebendazole and Fenbendazole against *Trichomonas vaginalis* and *Lamblia intestinalis* has been proved /3/. Albendazole has a clinical use in Lambliosis caused by some geographical strains of Lamblia intestinalis. There is experimental evidence of the antiprotozoan activity of benzimidazole compounds against Plasmodium falciparum /4/. Some bis-benzimidazoles are studied for antiprotozoal activity on *Entamoeba histolytica, Lamblia intestinalis, Trichomonas vaginalis* in culture /5/.

Synthesis and pharmaco-parasitological studies of novel benzimidazole compounds with antiprotozoal activity are promising and necessary due to the frequently registered resistance of some strains pathogenic parasites - intestinal, urogenital and blood protozoa.

Synthesized by us 33 novel benzimidazole compounds were tested for anthelmintic activity. Attention was focused on bis-benzimidazole compounds. According to parasitological publications, some of these compounds have antiprotozoal effect against *Lamblia intestinalis* with very good results /1, 5/. The aim of the present work is experimental investigation of the antiprotozoal effect of some novel synthesized bis-benzimidazole compounds with antihelmintic activity on *Trichinella spiralis* larvae.



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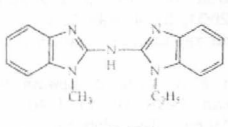

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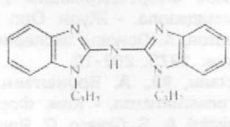

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The following tasks were formulated:

1. Establishing the "in vitro" effect of four novel synthesized bis-benzimidazoles on a culture of Paramaecium caudatum.
2. Experimental "in vivo" investigation of the effects of bis-benzimidazoles in white mice infected with Lamblia muris.

MATERIAL AND METHODS

1. Studied compounds. Four novel synthesized bis-benzimidazoles diluted in dimethylsufoxide /DMSO/ with concentration of 50 μg/ml:
2. Culture of Paramaecium caudatum obtained by soaking dry hay in water.
3. Experimental model of Lambliosis - 20 white mice infected with Lamblia muris.
4. Test - indicator for antiprotozoal activity "in vitro" on culture of Paramaecium caudatum. A drop diluted solution of each bis-benzimidazoleньs compounds was set in a small Petri-dish and mixed with several drops from the paramaecia culture. The reaction of *P. caudatum* was followed under a microscope /x400/.
5. A determinant of white mice infected with Lamblia muris. Fecal smears colored with Lugol solution were studied microscopically for the presence at protozoal cysts.
6. A test of antiprotozoal activity "in vivo". Infected with L. muris 20 white mice were divided into four grups with 5 mice each. They were treated "per os" for 2 days with 0,5 ml from each compound. Two days following treatment a control test of fecal smears for cysts of Lamblia muris was carried out.

RESULTS AND DISCUSSION

The anti-protozoal activity of the four bis-benzimidazoles was tested by us in an "in vitro" experiment on *Paramaecium caudatum*. Immediately after exposure the organismньs locomotion grew intense and chaotic, 2 minutes following exposure, the locomotion gradually slowed down, ceased and the cellular structure darkened. Vesicle-like projections appeared on the surface membrane, due to cell lysis. These phenomena prove the anti-protozoall activity of the tested bis-benzimidazoles on *P. caudatum*. Further "in vivo" experiments on white mice infested with Lamblia muris provided new evidence on the anti-protozoal activity of the tested bis-benzimidazoles. No L. muris cysts were identified in the feces of mice, following treatment. Lamblia trophozoites were found in the intestinal content of two of the mice from groop I. and in one of mice from each of the remaining groups. The experimental data show the antiprotozoal activity of the four tested bis-benzimidazoles against *P. caudatum* /"in vitro"/ and against *L. muris* /"in vivo"/.

The results of the study give us the grounds to consider conducting of further experiments on the protozoal activity of these compounds on larger numbers of experimental animals, infested with other protozoal parasites.

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CORRELATION BETWEEN FIVE METHODS FOR ANTIFUNGAL SUSCEPTIBILITY TESTING OF FLUCONAZOLE, VORICONAZOLE, ITRACONAZOLE, KETOCONAZOLE, 5-FLUOROCITOZINE AND AMPHOTERICINE B AGAINST CANDIDA SPP

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SUMMARY

Antifungal susceptibility testing may be an important aid in the treatment of patients with life-threatening yeast infections. The activity of Fluconazole, Itraconazole, Ketoconazole, Voriconazole, 5-Fluorocitosine, Amphotericine B against 71 clinical isolates of Candida spp. were tested by the E-test, disk diffusion, microdilulution method NCCLS M27-A2, ATB Fungus and Merlin. We determined comparatively high per cent of resistant to asole antifungals strains and good activity of Amphotericine B. The NCCLS method was found to be too complex and labor-intensive for routine testing. The agar-based E-test and disk diffusion methods are reliable alternative to the NCCLS M27-A2 reference microdilution method, but experience in determining MICs and careful attention to procedural details are critically important. Continued surveillance and refinement of broth - and agar-based test methods will help to identify susceptibility trends and improve the laboratory capability for antifungal susceptibility testing.

Key words: antifungal susceptibility testing, Candida spp., antifungals

Over the last two decades, the number of patients with serious fungal infections has increased dramatically. The populations at risk include patients with AIDS, those receiving cancer chemotherapy or organ transplantation, and others receiving immunosuppressive medications. The major agents of fungal infections are *Candida species*, and among them, *Candida albicans* is the most frequently isolated (17, 18) However as a consequence of the resulting extensive use of antifungal agents for treatment and prevention, a shift in the nature of infecting organisms has been reported (1, 4). Frequency of infections due to other yeast species such as Candida glabrata, *C. krusei, C. parapsilosis* and *C. tropicalis* is increasing. In several studies it was noted a potential for azole cross-resistance of some strains of *C. glabrata* and *C. krusei.* For the treatment of such clinically significant isolates is recommended antifungal susceptibility testing (6, 16).

There is an increased need of rapid, easy, reproducible, and inexpensive in vitro methods of obtaining susceptibility data which can guide the treatment of clinical yeast infections. The development of standardized antifungal susceptibility testing methods has been the subject of numerous studies during the last decade. Most of them are focused on the study of the susceptibility of yeasts from genus *Candida* to the most frequently used antifungals (Fluconazole, Itraconazole, Ketoconazole, Voriconazole, 5-Fluorocitosine, Amphotericine B) (7, 8).

National Committee for Clinical Laboratory Standards (NCCLS, present name-CLSI) has published an approved reference method for susceptibility testing of Candida and Cryptococcus spp. to five antifungals-NCCLS M27-A2 (9). This method is quite difficult, time and work consuming so this makes it inappropriate for routine laboratory diagnosis. Agar based susceptibility testing methods have been a focus of interest for many researchers and include the classical disk diffusion (DD) method NCCLS M44-A and E-test (ET) method. Those tests are very attractive due to their simplicity, reproducibility, and lack of requirements for specialized equipment (10, 11). In the clinical microbiology laboratories are also used commercial kits for antifungal susceptibility testing.

In the current study we determined the susceptibility to Fluconazole of Candida spp. strains by the DD method. Preliminary chosen resistant or with dose dependent susceptibility to Fluconazole strains were additionally tested for determination of their susceptibility to five antifungals: Itraconazole, Ketoconazole, Voriconazole, 5-Fluorocitosine, Amphotericine B. We used five methods microdilution method NCCLS M27-A2, DD method M44-A, E-test (AB BIODISK, Solna, Sweden) and the commercial kits ATB FUNGUS 2 INT (Bio Merieux, Marcy IнъEtoile, France) and MICRONAUT-AM Merlin (Merck, Germany). We compared results received with the different methods, determined the correlation between them and concerned their benefits and disadvantages.

MATERIALS AND METHODS

Isolates. In the study were used 71 clinical isolates collected by the Reference Mycology Laboratory of National Center of Infectious and Parasitic Diseases, Sofia. They were all *Candida sp.* strains: *C. albicans*-41, *C. parapsilosis*-8, *C. tropicalis*-2, *C. glabrata*-7, *C. krusei*-11, *C. zeylanoides*-1, *C. rugoza*-1. They were isolated from different clinical materials: blood, urine, vagina, sputum and throat, cardiac valve. Most of them were recovered from specimens from patients with AIDS. *C. parapsilosis* ATCC 22019 and C. krusei ATCC 6258 were included for quality control. All strains were identified by conventional biochemical methods API 20C AUX (Bio Merieux, Marcy l'Etoile, France), AUXACOLOR 2 (BioRad, Marnes la Coquette, France) or automated system Vitek (Bio Merieux, Marcy IнъEtoile, France) (3, 4). All strains were stored at -80°C, and prior to testing each isolate was subcultured twice on Sabouraud dextrose agar to ensure viability and purity.

Inoculum preparation. Prior to testing, each isolate was grown on Sabouraud agar for 24 h at 35°C. Suspensions were prepared in 0.85% saline to achieve a 0.5 McFarland standard by spectrophotometric measurement. The final concentration was approximately 1-5x10^6 CFU/ml. For ATB FUNGUS2 INT (Bio Merieux) we prepared inoculum suspension equivalent to 2 McFarland.

Disk diffusion method (NCCLS M44-A). For this method we used Mueller-Hinton agar with 2% glucose and 0.5xg/ml methylen blue dye (MH-GMB) according to the guidelines of NCCLS M44-A (11, 12). That medium produce enhanced definition of growth margins. The agar surface was inoculated by using a swab dipped in a cell suspension adjusted to the turbidity of a 0.5 McFarland standard by the Retro C80 (AB BIODISK, Solna, Sweden) device for rotational inoculation. A 25xg fluconazole-containing disk was placed on

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the surface of each plate by Nema C88 (AB BIODISK, Solna, Sweden) dispenser. The plates were incubated at 35°C, and zone diameters were read after 18 to 24 h. Zone diameter endpoints were read at 80% growth inhibition. The small and medium-sized colonies were considered nonresistant mutants, according to the manufacturerнъs guidelines.

NCCLS broth microdilution method (M27-A2). It was used for Fluconazole susceptibility testing. Following inoculation of the reference microdilution plates, they were incubated at 35°C in a non-CO_2 incubator, and MICs were determined after 24 and 48 h. Reference MICs corresponded to the lowest drug dilution that showed prominent (50% to 80%) growth inhibition. QC isolates were tested in the same manner (19).

E-test diffusion method. This method was used for determination of MICs to Fluconazole, Itraconazole, Voriconazole, Amphotericine B of the isolates. Etest strips were provided by AB BIODISK (Solna, Sweden). As in DDM, E-test was carried out on MH-GMB medium. The plates were inoculated as described above for DDM and were incubated at 35°C, and MICs were read after 24 h. The MICs were read as the lowest concentration at which the border of the elliptical inhibition zone intercepted the scale on the strip (20,21). Any growth such as microcolonies throughout a discernible inhibition ellipse was ignored.

MICRONAUT-AM MERLIN (Merck, Germany) colorimetric antifungal plate procedure. It consists of micro-titration plate for automated or manual susceptibility testing of yeasts (only for study purposes). This susceptibility testing is based on the rehydration of antimycotics by adding of standardized yeast suspension. Growth of the yeasts is indicated by a color change from blue to pink. MICRONAUT-AM enables MICs determination of Ketoconazole (0.031/0.25 xg/ml), Itraconazole (0.031/0.5 xg/ml), Fluconazole (1/16 xg/ml), Voriconazole (0.063/1 xg/ml), 5-Fluorocitosine (0.5?8 xg/ml) and Amphotericine B (0.063/1 xg/ml).

ATB FUNGUS2 INT (Bio Merieux, Marcy-lнъEtoile, France) commercial kit procedure. This strip enables the determination of MICs to Fluconazole, Itraconazole, 5-Fluocitosine, Amphotericine B of Candida and Cryptococcus neoformans in a semi-solid medium, under conditions similar to reference method NCCLS M27-A2.

Breakpoints. According to NCCLS M44-A method for Fluconazole susceptibility testing: 1) when the diameter is ?14 mm the strain is determined as resistant (R) with corresponding MIC?64 xg/ml; 2) diameter between 15-18mm-susceptible-dose dependent (S-DD) with corresponding MIC=16?32xg/ml; 3) diameter ?19mm-susceptible (S) strain with MIC?8xg/ml. The interpretation of the results received with the other methods is according to the interpretive breakpoints published by NCCLS.

Quality control

Two strains for quality control were tested concomitantly with the others in the same conditions. The control MICs were within the limits proposed in the M27-A2 document.

RESULTS AND DISCUSSION

Standardization of the methods for drug susceptibility testing is a very difficult process. The difficulties are connected with: type of inoculum, medium, time and temperature of incubation, determination and interpretation of the results, solubility of the drugs, variability of the yeasts and others. All this determines the necessity of parallel performance of several methods in order to achieve a comparative study of the results.

Table 1 shows the zone diameters and their corresponding interpretive categories determined by DDM for Fluconazole of 71 Candida strains. From 41 C. albicans strains 8 or 19.5% were determined as resistant. Such high pro cent of resistance requires verification with another method. This is not typical for *C. albicans*, which on the base of numerous studies is determined as comparatively susceptible to azoles (13). The high level of resistance can be explained with the fact that all resistant strains were isolated from patients with AIDS who had past long period of azole prevention and therapy (5, 14). The condition of permanent presence of antifungal in the environment allows the selection of resistant phenotype.

Table 1. Comparison of zone diameters and their corresponding interpretive categories in the Fluconazole susceptibility testing of 71 Candida strains.

Species (no. of isolates tested)	Categories and corresponding MICs (mg/ml)	Number of isolates	Zone diameters ranges (mm)
C. albicans-41	S, ≤8	16	19-37
	S-DD, 16-32	17	15-18
	R, ≥64	8	NZ-14
C. parapsilosis -8	S, ≤8	8	32-38
	-	-	-
	-	-	-
C. tropicalis-3	-	-	
	S-DD, 16-32	2	15-18
	R, ≥64	1	13
C. glabrata -7	S, ≤8	2	29-32
	S-DD, 16-32	3	15-16
	R, ≥64	2	NZ-12
C. krusei-11	-	-	-
	-	-	-
	R, ≥64	11	NZ-11
C. zeylanoides -1	-	-	-
	-	-	-
	R, ≥64	1	NZ
C. rugoza- 1	-	-	-
	-	-	-
	R, ≥64	1	NZ

S-susceptible, S-DD-susceptible-dose dependent, R-resistant, NZ-no zone, MIC-minimal inhibitory concentration.

Table 2. MICs data and interpretive categories of 15 isolates tested with MD, E-test, Merlin, ATB Fungus.

		Antifungals					
Strains	Methods	FLU	VOR	ITR	KETO	FCY	AMB
C. albicans	MD	R, 64	-	-	-	-	-
62	E-test	R, >256	R, >32	R, 1	-	-	S, 0.064
	Merlin	R, >128	R, 8	R, > 4	R, > 2	S, <0.5	S, 0.064
DD, R	Fungus	R, 64	-	R, 4	-	S, <0.5	ND, <0.5
C. albicans	MD	R, 64	-	-	-	-	-
69	E-test	R, >256	R, >32	I, 0.75	-	-	S, 0.063
	Merlin	R, >128	R, 8	R, > 4	R, >2	S, <0.5	ND, 0.5
DD, R	Fungus	R, 64	-	R, 4	-	S, <0.5	ND, <0.5
C. krusei	MD	R, 64	-	-	-	-	-
616	E-test	R, >256	ND, 0.75	R, 2	-	-	ND, 1
	Merlin	R, >128	ND, 1	R, 1	R, 1	I, 16	ND, 5
DD, R	Fungus	R, 64	-	R, 2	-	I, 8	ND, <0.5
C. glabrata	MD	S, 1	-	-	-	-	-
621	E-test	S-DD, 32	S, 0.25	R, 1.5	-	-	ND, 0.38
	Merlin	R, 64	S, 0.125	R, 1	ND, 0.125	S, <0.5	ND, 0.5
DD, SDD	Fungus	S, 4	-	R, 2	-	S, <0.5	ND, <0.5
C. albicans	MD	S, 4	-	-	-	-	-
639	E-test	S, 12	S, 0.125	S-DD, 0.25	-	-	S, 0.064
	Merlin	S, 8	S, 0.125	S-DD, 0.25	ND,0.125	S, <0.5	ND, 0.5
DD, SDD	Fungus	S, 8	-	I, 0.25	-	S, <0.5	ND, <0.5
C. glabrata	MD	S, 1	-	-	-	-	-
697	E-test	S-DD, 32	ND, 0.5	I, 0.75	-	-	ND, 0.38
	Merlin	S, 4	S, 0.125	S-DD, 0.75	ND,0.125	S, <0.5	ND, 0.5
DD, SDD	Fungus	S, 2	-	S-DD, 0.5	-	S, <0.5	ND, <0.5
C. krusei	MD	S-DD, 16	-	-	-	-	-
698	E-test	S-DD, 32	ND, 0.5	R, 1	-	-	ND, 0.125
	Merlin	R, 64	ND, 0.5	R, 1	R, 1	R, 16	ND, 1
DD, R	Fungus	S-DD, 32	-	R, 1	-	R, 8	ND, <0.5
C. tropicalis	MD	S, 1	-	-	-	-	-
6173	E-test	R, >256	ND, 0.5	S-DD, 0.25	-	-	S, 0.094
	Merlin	S, 8	ND, 0.75	S-DD, 0.25	S, 0.031	S, <0.5	ND, 0.5
DD, R	Fungus	R, 64	-	R, 1	-	S, <0.5	ND, <0.5
C. albicans	MD	S, 1	-	-	-	-	-
6115	E-test	S, 4	S, 0. 19	S, 0.125	-	-	ND, 0.19
	Merlin	S, 1	S, <0.063	S, <0.031	ND, 0.125	S, <0.5	ND, 0.5
DD, R	Fungus	S-DD, 16	-	S-DD, 0.5	-	S, <0.5	ND, <0.5
C. rugosa	MD	S-DD, 32	-	-	-	-	-
6190	E-test	R, >256	ND, 0.75	I, 0.75	-	-	ND, 1
	Merlin	S-DD, 32	ND, 1	S-DD, 0.5	ND, 0.5	I, 16	ND, 1
DD, R	Fungus	S-DD, 32	-	S-DD. 0.5	-	I, 8	ND, =0.5
C. glabrata	MD	S, 1	-	-	-	-	-
6224	E-test	R, 128	R, >32	R, 1.5	-	-	ND, 1
	Merlin	S, 8	ND, 1	S-DD, 0.5	ND, 0.25	S, <0.5	ND, 0.5
DD, R	Fungus	S, 4	-	R, 1	-	S, <0.5	ND, <0.5
C. glabrata	MD	S, 2	-	-	-	-	-
6228	E-test	S-DD, 32	ND, 0.75	R, 6	-	-	ND, 0.25
	Merlin	S-DD, 16	ND, 1	R, 2	ND, 0.5	S, <0.5	ND, 0.5
DD, R	Fungus	S, 2	-	S-DD, 0.25	-	S, <0.5	ND, <0.5
C. glabrata	MD	S, 4	-	-	-	-	-
6386	E-test	R, >256	ND, 3	R, >32	-	-	ND, 0.25
	Merlin	S-DD, 16	S, 0.25	R, 1	ND, 0.25	S, <0.5	ND, 0.5
DD, R	Fungus	S-DD, 16	-	R, 2	-	S, <0.5	ND, <0.5
C. glabrata	MD	S, 1	-	-	-	-	-
2331B	E-test	S-DD, 16	S, 0.25	R, 1.5	-	-	ND, 0.38
	Merlin	S, 8	S, 0.25	R. 1	ND, 0.25	S, <0.5	ND, 0.5
DD, R	Fungus	S, 4	-	S-DD, 0.5	-	S, <0.5	ND, <0.5
C. parapsilosi	MD	S, 0.5	-	-	-	-	-
ATCC 22019	E-test	S, 1	S, 0.032	S, 0.047	-	-	ND, 0.19
	Merlin	S, 1	S, <0.063	S, <0.031	S, <0.031	S, <0.5	ND, 0.25
DD, S	Fungus	S, 0.5	-	S, <0.125	-	S, <0.5	ND, <0.5

MD-micro-dilution method ; DD-disk diffusion; S-susceptible; I-intermediate; S-DD-susceptible dose-dependent; R-resistant; ND-not defined; FLU-Fluconazole; VOR- Voriconazole; ITR-Itraconazole; KETO-Ketoconazole; FCY-Fluorocitosine; AMB-Amphotericine B.

In the study was confirmed, the expected high susceptibility to Fluconazole of C. parapsilosis isolates. Strains of C. glabrata and C. krusei which are innately resistant to Fluconazole, as described in the literature, with DDM were confirmed as isolates with low susceptibility (22, 23). Some of the strains of C. tropicalis, C. zeylanoides and C. rugoza, were also determined as resistant. From all 71 isolates 23 or 32.3% were determined as resistant. Disk-diffusion method is very attractive due to its simplicity, reproducibility, and lack of requirements of specialized equipment.

The biggest problem connected with MD method performance was so called trailing growth with some strains, which is result of partial growth inhibition by Fluconasole. The high pro cent of Fluconazole resistance induced us to investigate preliminary chosen strains with four more methods and other antifungals. In table 2 are snowed the MICs and their corresponding interpretive categories of 15 isolates. When we compared the Fluconazole susceptibility with five methods we concluded that: 1) for the C. albicans and C. krusei isolates there is good correlation between DDM and the other methods; 2) C. glabrata strains determined as S-DD and R were not confirmed; 3) there is good correlation between DDM and E-test, but the received MICs were higher compared to the other methods 4) comparatively good correlation was determined between microdilutional method and ATB Fungus-with the exception for C. tropicalis; 5) despite of the different MICs determined with the five methods, strains were related to one and the same category.

In May 2002, Voriconazole was approved by the USA Food

and Drug Administration (FDA) for primary treatment of aspergillosis and salvage therapy in cases of serious infections caused by unusual molds such as Fusarium and/or Scedosporium species. In Europe, it has also been approved for treatment of severe yeast infections, including those due to C. glabrata and C. krusei. In the current study Voriconazole susceptibility was determined by E-test and commercially available MICRONAUT-AM kit. In resistant strains we determined higher MICs with E-test than with MICRONOUT-AM and the correlation between the two methods is bigger with more susceptible strains.

We had studied the Itraconazole susceptibility with tree methods E-test, Merlin and Fungus. We obtained good coincidence of the results of the resistant strains, with just a little variation of the MICs. There were some discrepancies with dose dependent susceptible strains. Itraconazole showed not very good activity against the tested isolates.

Ketoconazole susceptibility was determined only by Merlin. For that antifungal agent there are no interpretive categories for yeasts but most of Candida sp. strains with MIC>0.125xg/ml are not susceptible to Ketoconazole therapy. For the tested strains the MICs were in the range of 0.125 to >2xg/ml, so they had low susceptibility to Ketoconazole.

From the results published in Table 2, we can talk about a common Hbbehaviourh of the strains to each one of the azole antifungals. Most of the isolates, which were resistant or susceptible to a certain azole antifungal, refer to others the same way. The reasons for that common "behaviour" are investigated on molecular level.

The antifungal agent 5-Fluorocitozine acts as causing mistakes in protein synthesis and influences nucleic acids in the yeast cells. In this study we determined that it had a very good activity to azole resistant strains. With the exception of C. rugosa and C. krusei isolates, that showed lower susceptibility. The coincidence received with the two methods MICRONAUT-AM and ATB Fungus was very good.

The polyenic antifungal Amphotericine B attacks the ergosterol in the cell membrane of the fungi. In contrast to azoles witch are fungistatic, it has a fungicidic action on the fungal cells. So this antifungal is appropriate for treatment of infections caused by resistant to azoles fungi, but it is very toxic. For Amphotericine B there are no proposed MICs limits witch can be used for clinical interpretation, but strains with MIC>1xg/ml are probably resistant. Very good correlation was determined for the MICs received with Merlin and Fungus, while E-test MICs were lower. Higher values of the MICs were detected for C. krusei and C. rugosa isolates which were probably resistant. The results obtained by both the DD and the ET methods were in acceptable concordance with those obtained by the MD method, with the exception of the recurring problem of discrepancies due to isolates that showed trailing growth. In summary, the agar-based ET and DD methods are reliable alternatives to the NCCLS M27-A2 reference MD method for isolates that test susceptible to fluconazole. However, the detection of resistance by agar-based methods correlates poorly with the detection of resistance by the reference NCCLS M27-A2 method. Although more work needs to be done with less susceptible isolates, the aggregate data suggest that agar-based methods appear to produce a more consistent in vitro-in vivo correlation than the reference MD method by eliminating trailing growth from the equation.

During the last few years all the efforts are directed to the improvement of the existing and introduction of new methods for antifungal susceptibility of medically relevant yeasts. Standardized methods for culture and in vitro antifungal susceptibility testing with high intra-laboratory and inter-laboratory reproducibility can also provide reliable data for identifying local patterns, which must be taken into account in the selection of empiric therapy, especially in cases of severe candidiasis or candidemia (15). That can help clinicians in identifying antifungal drugs with a higher probability of therapeutic efficacy.

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COMPARATIVE STUDY OF LABORATORY METHODS FOR IDENTIFICATION OF MEDICALLY IMPORTANT YEASTS

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SUMMARY

Advances in antifungal therapy necessitate the need for accurate identification of fungi, especially yeasts to their species level for more effective management. The identification of yeasts is best accomplished using a combination of colonial, microscopic and biochemical methods. The colonial morphological features are variable and often are not helpful. The microscopic morphological features of yeasts are helpful, but don't give 100% correct identification. Biochemical methods include fermentation and assimilation patterns of various carbohydrates, the new commercialized yeast identification systems are simpler, rapid and are particularly easy to interpret.

Key words: yeasts, Candida, morphological identification, biochemical methods for identification (Api, Aux, Fongiscreen, Vitek, Merlin)

Laboratory methods for identification of medically important yeasts are based on the study different phenotypic properties of the yeasts that include colony morphology and microscopic morphology, sugar assimilation and fermentation reactions. The various commercial identification systems introduced for yeast identification are based on color changes denoting the utilization of the several kinds of substrates by the metabolizing yeast. These methods are different.

The rate of correct identification is based on macroscopic, microscopic and biochemical methods (8).

MATERIALS AND METHODS

In the present study were used referent strains and isolates from clinical specimens:

Referent strains	Clinical isolates
Candida albicans ATCC 60193	Candida albicans
Candida tropicalis B6030413	Candida rugosa
Candida krusei 70075	Candida krusei
Candida glabrata ATCC 90050	Candida lusitaniae
Candida guilliermondii 9058	Trichosporon spp.
Candida parapsilosis 458	Cryptococcus neoformans

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ABBREVIATIONS USED IN THIS PAPER:
CA-Chrom Agar

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These strains were identified using morphological and biochemical tests.

Morphological tests include:

* Corn meal agar (MERCK, Germany) - the purpose is to determine the microscopic morphology. The incorporation of Tween 80 into Corn meal agar reduces the surface tension of the medium to promote the germination and sporulation of yeasts. After incubation for 48 h at 30°C, observe of a bright field microscope for the presence of hyphae, pseudohyphae, blastoconidia, arthroconidia or chlamydospores (13).

* Chrom agar - CA (Becton Dickinson,Cockeysville, USA) - it is chromogenic media that contains various substrates for the enzymes of yeast species.

CA is reported to give green colonies of C. albicans and blue colonies of C. tropicalis. C. krusei isolates produce pink colonies (15).

* Rapid identification Germ tube test - it is a rapid screening test where the production of germ tubes within 2 - 4 hours in contact with serum is considered as indicative of C. albicans. This test has to be confirmed with Corn meal agar test.

The procedure include suspending a very small inoculum of yeast cells in a 0,5 ml sterile serum (normal human serum, sheep serum or fetal calf serum). After incubation at 37°C for 2 -4 h, have to make microscope slide. Germ tube appears as filaments, which are not constricted at their point of origin on the parent cell (9).

Biochemical methods are different - commercial nonautomated tests (Api 20CAux, Auxacolor, Fongiscreen) and commercial automated identification systems (Vitek, Merlin, mini Api).

API 20 C Aux (BioMerieux, 69280 Marcy-l'Etoile, France) is a system for the precise identification of the most frequently encountered yeasts.

The API 20 C Aux strip consists of 20 cupules containing dehydrated substrates that enable the performance of 19 assimilation tests. The cupules are inoculated with a semi-solid minimal medium and the yeasts will only grow if they are capable of utilizing each substrate as the sole carbon source (14).

The reactions are read by comparing them to growth controls and identification is obtained by referring to the Analytical Profile Index or using the identification software.

Identification is performed using the database - have to look up the numerical profile in the list of profiles (table 1).

There is API Candida (BioMerieux, France) - a standardized system for the identification in 18 - 24 h of yeasts, notably those most frequently encountered in clinical microbiology.

The AUXACOLOR (BioRad, 92430 Marnes la Coquette, France) kit is identification system based on the principle of sugar assimilation. The growth of yeasts is visualized by the color change of a pH - indicator - from blue to yellow or green-yellow - it is positive test. The kit also contains 3 enzyme tests, including a test for detection of the phenoloxidase activity of Cryptococcus neoformans (7).

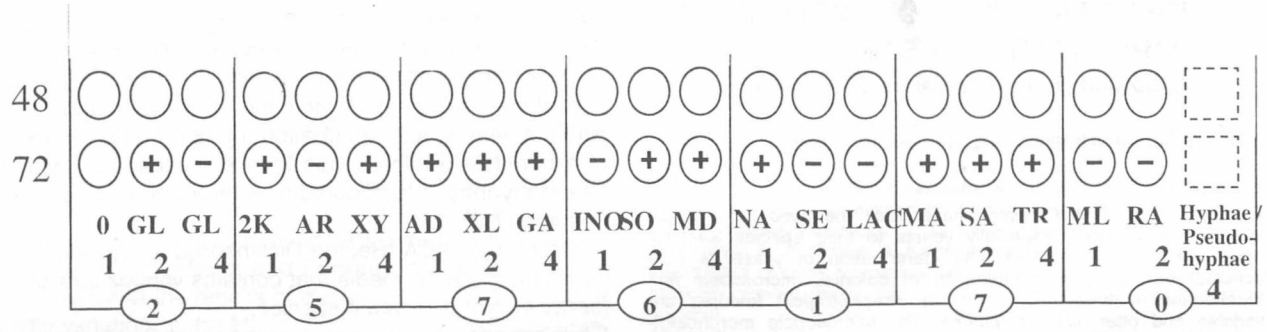
The final identification is based on a combination of the biochemical tests and complementary criteria, established under the usual conditions. The numerical profile must be determined from the list (table 2).

FONGISCREEN (BioRad, 92430 Marnes la Coquette, France) is based on the use of dehydrated substrates by fungal enzymes, resulting in a color change either spontaneously or after addition of a revelator reagent.

The obtained profile has to compare with the specific profile. This test can be used for identification of *C. albicans, C. glabrata, C. tropicalis, Cr. neoformans* and orientation of the

Table 1. API 20 C Aux

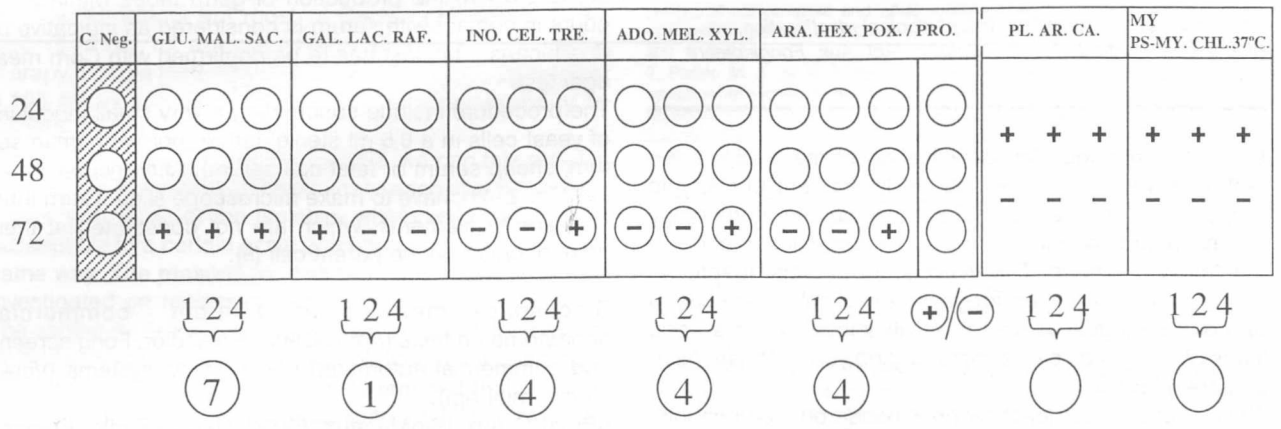
Api C 20



2576170- C. albicans

Table 2. Auxacolor

Auxacolor



71444 C. albicans

Table 3.

№	GAL	XYL	XLT	ERY	2KD	LAC	ARA	DUL	MEL	URE	STU	TRE	ADO	CYC	48H	MLT	MTZ	PLA	GLU	CEL	RAF	GLY	INO	AMG	NAG	SOR	NIT	VITEK-identification
1	+	+	+	-	+	-	-	-	-	-	+	+	+	+	-	+	-	+	+	-	+	+	-	+	+	+	-	C.albicans
2	+	+	-	-	+	-	+	-	-	+	+	+	+	-	-	+	+	+	+	-	-	+	-	+	+	+	-	C.parapsilosis

Table 4. Characteristics of commercial yeast identifications systems

Characteristics	Api 20 C	Auxacolor	Fongiscreen	Mini Api	MERLIN	VITEK
incubate time	48 h	48 h	4 h	24-48 h	24-48 h	24-48 h
temperature	30°C	30°C	37°C	35°C	30°C	30°C
McFarland	2	1.5	6	2	0.5	2
suspension medium	ApiC- suspension medium	suspension medium	suspension medium	dH_2O	dH_2O	0,45% NaCl
reading of the results	visually numerical profile	visually numerical profile	visually table	automatic software	automatic software	automatic software
interpretation						

diagnosis for other yeasts.

The VITEK (BioMerieux, 69280 Marcy-lЛъEtoile, France) is automated system for identification of the most frequently isolated clinical bacteria and yeasts.

Final organism identification may be reported after 24 or 48 hours incubation at 30°C and a single reading of the YBC CARD in the Vitek Reader/ Incubator. The biochemical test results are matched to the React File (date base). The React File is a matrix of percent probabilities of positive test for each biochemical test and each organism identified by the YBC CARD (example table 3).

MERLIN Micronaut (MERCK, Germany) is a system for automated identification and susceptibility testing of bacteria and yeasts.

The Micronaut plate contains dried substrates selected to assure accurate identifications. The plates are read using the Skan software, that provides an extensive identification data base (14).

Mini API (BioMerieux, France) is automated microbiology system for identification, based on biochemical tests in a specially microplate.

Table 5. Diagnostic abilities on the different tests for yeast identification

Species	Corn meal agar	Chrom agar	Germ tube test	Api 20C	Auxacolor	Fongiscreen	Mini Api	MERLIN	VITEK
C. albicans	+	+	+	+	+	+	+	+	+
C. tropicalis	+	+		+	+	+	+	+	+
C. krusei	+	+		+	+		+	+	+
C. glabrata	+	+		+	+	+	+	+	+
C. parapsilosis	+	+		+	+		+	+	+
C. guilliermondii				+	+		+	+	+
C. lusitaniae				+	+		+	+	+
C. rugosa				+	+		+	+	+
Cr. neoformans	+			+	+	+	+	+	+
Trichosporon spp.	+			+	+	+	+	+	+

RESULTS AND DISCUSSION

The methods for identification have different characteristics: incubate time, temperature, McFarland, suspension medium, interpretation (table 4).

Morphological and biochemical tests have some disadvantages and limits (12).

The use of Chrom agar is easy and reliable method for the presumptive identification of most commonly isolated Candida spp., especially C. albicans, C. tropicalis and C. krusei, but sometimes colonies morphology is hardly differentiation.

The presence of a true germ tube is characteristic of C. albicans, however have a pseudo-germ tube of C. tropicalis. Germ tube negative yeasts isolated should be identified to species level, for which the sample must be referred to the Reference Laboratory (10).

The Api 20 C and Auxacolor are systems for identification of yeasts, included in the database. In cannot be used to identify any other microorganisms, and only pure cultures of a single organisms should be used. Exceptionally, positive sugar assimilation tests may lose their color as the wells become pale colorless.

Fongiscreen system is able to identify for 4 hours, but limit number yeasts (*C. albicans, C. glabrata, C. tropicalis, Cr. neoformans*).

The VITEK - automated microbiology system give an identification report, which is printed for each CARD, placed in the Reader/ Incubator. Each report contains a card identification number, time, data, species and probability.

The excellent performance of MERLIN systems has been proved in routine laboratory diagnostics, clinical studies for evaluation of drugs of new antimicrobials and antimicrobial resistance surveillance (2,6).

Comparative study of yeast identification methods shows that, the best diagnostic abilities are automated microbiology system - they are effective, rapid, reliable and have experience in antimicrobial susceptibility testing (1, table 5).

The rate of correct identification must be based on the combination of morphological and biochemical methods for determination of medically important yeasts that can be routinely used in clinical laboratories.

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PRELIMINARY CHARACTERIZATION OF THE TYPES OF BETA-LACTAMASES PRODUCED BY CEFTAZIDIME-RESISTANT PSEUDOMONAS AERUGINOSA STRAINS

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SUMMARY

Objective: To preliminary characterize the main types of beta-lactamases among ceftazidime-resistant strains of *P. aeruginosa* and to determine their rate of resistance. Materials and methods: A total of 30 ceftazidime (CAZ)-resistant P. aeruginosa strains were collected from four large hospitals in Sofia during 2001-2005. Antimicrobial resistance towards 17 antimicrobial agents among the selected strains has been surveyed according to NCCLS-2003. The antibacterial activity of some beta-lactams, aminoglycosides and ciprofloxacin has been tested by determination of their minimum inhibitory concentrations (MICs) using Etest (AB Biodisk). The prevailing resistance mechanisms were studied as previously described by Jarlier et al. and Lee et al. Isoelectric points (pIs) of beta-lactamases produced by these strains were determined by isoelectric focusing (IEF) according to Mathew/Bauernfeind. The hydrolytic activity of the bands was proved by Bioassay (Bauernfeind). Results: The rates of resistance were: to carbenicillin- 100 %, azlocillin- 96.7 %, piperacillin- 93.3 %, piperacillin/tazobactam - 56.7 %, ceftazidime - 100 %, cefoperazone - 100 %, cefepime - 100 %, cefpirome - 100 %, aztreonam - 96.7 %, imipenem - 56.7 %, meropenem - 56.7 %, amikacin - 93.3 %, gentamicin - 93.3 %, tobramycin - 96.7 %, netilmicin - 76.7 %, ciprofloxacin - 90 % and polymyxin B - 0 %. MICs of CAZ ranged from 32 to >256 mg/l. 14 strains of all 30 CAZ-resistant *P. aeruginosa* (46.7 %) were presumptive producers of extended-spectrum beta-lactamases (ESBLs) according to the double disk synergy test of Jarlier et al. 12 strains (40 %) were resistant to all beta-lactams, including to carbapenems, but they did not show a positive Hodge test for metallo-beta-lactamases. The strains demonstrated four different beta-lactamases with pIs: 5.7; 6.1; 7.4 and 8.2. 25 strains of all studied CAZ-resistant P. aeruginosa (83.3 %) expressed CAZ hydrolytic bands with pI value of 7.4 (positive Bioassay) likely corresponded to the pI of VEB-1 ESBL. The other presumptive beta-lactamases were: CARB-enzymes (pI 5.7), OXA-enzymes (pI 6.1) and AmpC cephalosporinase (pI 8.2). In 5 from investigated 30 strains (16.7 %) resistance to CAZ was associated with the overproduction of chromosomal AmpC beta-lactamase, or with non-enzymatic mechanisms such as drug efflux or outer membrane impermeability. Conclusion: In the present study resistance to extended-spectrum cephalosporins, including to CAZ, was related to the production of VEB-1 type ESBL from Ambler class A. VEB-1 is widespread mostly in Asia and is rarely in European countries. Nevertheless it appears to have a significant presence among CAZ-resistant P. aeruginosa isolates in Bulgarian hospitals and causes serious impediments in antimicrobial treatment.

Key words: Pseudomonas aeruginosa, ceftazidime resistance, beta-lactamases, isoelectric focusing.

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ABBREVIATIONS USED IN THIS PAPER: ESBLs- extended-spectrum beta-lactamases, IEF- isoelectric focusing, pIs- isoelectric points, CAZ- ceftazidime, FEP- cefepime, IMP- imipenem, MER- meropenem, AMK- amikacin, GEN- gentamicin, CIP- ciprofloxacin, MIC- minimum inhibitory concentration, DDST- double disk synergy test, NCCLS- the National Committee for Clinical Laboratory Standards, R- resistant.

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Pseudomonas aeruginosa is one of the bacterial species most frequently responsible for nosocomial infections and is notably resistant to many antibiotics, including beta-lactams (8). This pathogen is characterized by its natural resistance to beta-lactam agents and its ability to acquire additional mechanisms of resistance to these drugs (5). The main mechanisms of acquire resistance include: overproduction of the derepressed AmpC cephalosporinase that confers resistance to all beta-lactams with the exception of carbapenems (29); production of transferable beta-lactamases from different molecular classes and non-enzymatic mechanisms such as overexpression of the MexA-MexB-OprM efflux system mainly affecting the activities of carboxypenicillins and monobactams (35) or porin D2 deficiency leading to carbapenem resistance (28). The classical beta-lactamases belonging to the CARB, OXA or TEM group are associated with a penicillinase production phenotype (resistance to carboxypenicillins and ureidopenicillins and susceptibility to ceftazidime) (4, 5), whereas the extended-spectrum beta-lactamases (ESBLs) from Ambler class A (PER-, VEB-, GES-/IBC-, TEM- and SHV-type), class D (extended-spectrum oxacillinases- point mutant derivates of OXA-10 or of OXA-2; and OXA-18) and class B (metallo-beta-lactamases such as IMP-, VIM- and SPM-type) are capable of hydrolyzing extended-spectrum cephalosporins (5, 9, 26, 27). In contrast to the enterobacterial species in which TEM- and SHV-type enzymes are most frequent, OXA- and PSE (Pseudomonas specific enzyme)-types are the most frequently encountered beta-lactamases in *P. aeruginosa* (18, 34).

This study was performed to preliminary characterize the main types of beta-lactamases among ceftazidime-resistant strains of *P. aeruginosa* and to determine their rate of resistance using phenotypic methods and isoelectric focusing.

MATERIALS AND METHODS

Bacterial strains. 30 strains of *P. aeruginosa* determined by the disk diffusion method on Mueller-Hinton agar according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines (24) as ceftazidime-resistant (zone diameter, < 18 mm) were investigated. The strains were collected from four large hospitals in Sofia during 2001-2005 and were stored at -70°C in 20% skimmed milk until used in this study. Isolates were from different clinical specimens: urine - 12, bronchopulmonary sample - 8, wound- 6, nose swab - 2, throat swab - 1, and rectal swab- 1. The identification was performed by standard microbiological methods (tests for cytochrome oxidase, arginin dihydrolase, nitrate reduction, growth at 42 (C) and was confirmed by NEFERMtest 24 (LA CHEMA).

Antimicrobial susceptibility (AS) and Minimum inhibitory concentrations (MICs) determinations. AS towards 17 antimicrobial agents among the selected strains has been surveyed according to the recommendations of the NCCLS-2003 using the disk diffusion method (24). The used medium was Mueller-Hinton II agar (Becton Dickinson, BBL, USA) and the following antimicrobial disks, provided by NCIPD (Sofia, Bulgaria) or BBL (USA), were used: carbenicillin (CAR)-100 μg/disk, azlocillin (AZL)-75 μg, piperacillin (PIP)-100 μg, piperacillin/tazobactam (TZP)-100/10 μg, cefoperazone (CPZ)-75, ceftazidime (CAZ)-30, cefepime (FEP)-30, cefpirome (CPO)- 30, aztreonam (ATM)-30, imipenem (IMP)-10, meropenem (MER)-10, amikacin (AMK)-30, gentamicin (GEN)-10, netilmicin (NET)-30, tobramycin (TOB)-10, ciprofloxacin (CIP)-5, and polymyxin B (PB)- 300 UI.

The MICs of selected beta-lactams (CAZ, FEP, IMP, MER), aminoglycosides (AMK and GEN) and CIP were determined

using Etest (AB Biodisk, Solna, Sweden) on Mueller-Hinton II agar plates according to the manufacturerьs instructions (6). All plates were incubated at 35°C for 18 h. MIC results were interpreted according to NCCLS guidelines (24). *P. aeruginosa* ATCC 27853 was used for quality control purposes in susceptibility testing.

Phenotypic methods for detection of resistance mechanisms to antimicrobial agents:

Detection of extended-spectrum beta-lactamases (ESBLs). Strains were screened for the presence of ESBLs by the double-disk synergy test (DDST) as described by Jarlier et al. (14). CAZ (30 μg), FEP (30 μg), CPO (30 μg) and ATM (30 μg) disks were placed 25 mm (center to center) from the TZP (100/10 ?g) disk on Mueller-Hinton agar plate inoculated with the test organism, which was adjusted to the McFarland № 0.5 tube. After overnight incubation, enhancement of the inhibition zone around one or more of these disks toward the tazobactam-containing disk indicated the presence of ESBLs.

All studied ceftazidime-resistant strains were additionally tested by double-disk diffusion method with carbapenem (IMP) and 3. generation cephalosporin (CAZ) for the presence of synergism.

Detection of metallo-beta-lactamases (MBLs). The presence of MBLs from molecular class B (16) was studied by the modified Hodge test (17). The indicator organism, E. coli ATCC 25922, at a turbidity of 0.5 McFarland standard, was used to swab inoculate the surface of a Mueller-Hinton agar plate, and the test strain was heavily streaked from the center to the plate periphery. After the plate was allowed to stand for 15 min at room temperature, an IPM disk (10 ?g) was placed at the center, and the plate was incubated overnight. The presence of a distorted inhibition zone was interpreted as a positive result for carbapenem hydrolysis screening.

Beta-lactamase preparation. Cultures of CAZ-resistant *P. aeruginosa* were grown overnight at 37°C in 100 ml Tryptic Soy Broth (Difco) containing CAZ, 1mg/l with continuous shaking. The cells were separated with centrifugation, then were disrupted by sonication (75 pulses/min for 3 min at 20 KHz) and centrifuged (14 000 rpm for 30 min, 4°C). The supernatant contained the crude enzyme extract.

Analytical isoelectric focusing. Crude beta-lactamase extracts were subjected to isoelectric focusing (IEF) in a 30 % polyacrylamide gel containing ampholyte (pH range, 3 to 10) Isolyte (ISN, Germany) by the modified method (3) of Mathew et al. (22). The regimen included: max 10 mA, 1000 V, 2 h. The focused enzymes were visualized by overlaying the gel with 0.5 mM nitrocefin (Oxoid, UK) and isoelectric points (pIs) were estimated by comparison with TEM-3, SHV-3, SHV-5 and CMY-2 standards.

Bioassay. After IEF, the ceftazidime (CAZ)-hydrolyzing activity was assigned to particular beta-lactamases by bioassay, as described by Bauernfeind et al. (3). The IEF gel was overlaid with Tryptic Soy Agar (50 %) containing CAZ, 2 mg/l and incubated at 35°C for 2 h. Then it was overlaid with TSA 50 % containing E. coli ATCC 25922, 1.2 x 109 CFU/ml. After overnight incubation at 35°C the presence of growth of the clinical strain determined the bands hydrolyzed CAZ.

RESULTS AND DISCUSSION

The rates of antimicrobial resistance of the studied ceftazidime-resistant *P. aeruginosa* strains were: to carbenicillin - 100%, azlocillin - 96.7%, piperacillin - 93.3%, piperacillin/tazobactam - 56.7%, ceftazidime (CAZ) - 100%, cefoperazone- 100%, cefepime (FEP)- 100 %, cefpirome- 100%, aztreonam- 96.7%, imipenem (IMP) - 56.7%, meropenem (MER)- 56.7%, amikacin (AMK)- 93.3%, gentamicin (GEN) - 93.3%, tobramycin - 96.7%, netilmicin - 76.7 %, ciprofloxacin (CIP)- 90 % and polymyxin B- 0 % (table 1). The selected strains were a part from a collection of problematic clinical isolates of *P. aeruginosa* obtained from 4 large hospitals in Sofia during 2001-2005. For these problematic strains (resistant to cephalosporins, 3rd/4th generation; carbapenems; aminoglycosides or/and fluoroquinolones) CAZ resistance rate was 26.6 % (32), similar to the average resistance rate to this antibiotic (24.5 %) in Bulgaria for 2003 (30). It was lower than that in countries such as Brazil and Turkey, but higher than the CAZ resistance rate in USA at the same time. In 1999-2002 in some Turkish University Hospitals the incidence of CAZ-resistant P. aeruginosa varied from 35 to 51 % (11). In Brazil the established average CAZ resistance rate for 2003 was 36 % according to data of the MYSTIC Program Brazil 2003 (15). In 1999-2002 the frequency of CAZ-resistant strains of *P. aeruginosa* in USA was 13 % according to TSN Database-USA (Focus Technologies, Herndon) (10).

The antibacterial activity (AA) of the following antibiotics-CAZ, FEP, IMP, MER, AMK, GEN and CIP, against the investigated strains of P. aeruginosa was tested by their minimum inhibitory concentrations (MICs) using Etest (6). The determined MICs-rates (mg/l), including MIC50, MIC90 and MIC range, are presented in table 2. MICs (mg/l) of CAZ, FEP, IPM, MER, AMK, GEN and CIP against P. aeruginosa ATCC 27853 were respectively 1; 2; 1; 0.5; 1; 0.5 and 0.5 and determined P. aeruginosa ATCC 27853 as a susceptible strain towards CAZ, FEP, carbapenems, aminoglycosides and CIP according to NCCLS-2003 (24).

The MICs of CAZ were in a wide range from 32 to >256 mg/l. 90 % of the studied strains were inhibited by concentration of this antibiotic more than 256 mg/l. For comparison, in a recent prospective survey of beta-lactamases produced by CAZ-resistant *P. aeruginosa* in France the MICs of CAZ were in a narrow range from 32 to 128 mg/l (9). Our strains were resistant (R) to FEP (MICs were more than 32 mg/l). Resistance to both IMP and MER was observed in 13 isolates (43.3 %) of all tested *P. aeruginosa*. IMP-resistance in *P. aeruginosa* typically reflects reduced uptake as a result of loss of the OprD porin (20) and resistance by this mechanism codepends on continued expression of the chromosomal Amp C beta-lactamase (19). Resistance to MER, but not to IMP, can arise via overexpression of the MexA-MexB-OprM active efflux system (20, 21). In this study MER showed slight higher AA (40 % of susceptible strains) than IMP (33.3 %) against included CAZ-resistant strains of *P. aeruginosa*. The MICs of MER ranged from 0.190 to >32 mg/l and these

Table 1. Antimicrobial resistance among 30 ceftazidime-resistant strains of Pseudomonas aeruginosa according to the Disk diffusion method (NCCLS, 2003).

Antimicrobial agents	Number (%) I+R strains
Carbenicillin	30 (100)
Azlocillin	29 (96.7)
Piperacillin	28 (93.3)
Piperacillin+Tazobactam	17 (56.7)
Cefoperazone	30 (100)
Cefepime	30 (100)
Cefpirome	30 (100)
Aztreonam	29 (96.7)
Imipenem	17 (56.7)
Meropenem	17 (56.7)
Amikacin	28 (93.3)
Gentamicin	28 (93.3)
Tobramycin	29 (96.7)
Netilmicin	23 (76.7)
Ciprofloxacin	27 (90.0)
Polymyxin B	0 (0)

I- intermediate susceptible, R- resistant.

Table 2. Antimicrobial activities of 7 antimicrobial agents against 30 ceftazidime-resistant strains of Pseudomonas aeruginosa determined using Etest.

Antimicrobial agent	MIC range (mg/l)	MIC_{50} (mg/l)	MIC_{90} (mg/l)	Number (%) of resistant strains	MIC against *P. aeruginosa* ATCC 27853
Ceftazidime	32 - >256	>256	>256	30 (100)	1
Cefepime	>32	>32	>32	30 (100)	2
Imipenem	1 - >32	8	>32	14 (46.7)	1
Meropenem	0.190 - >32	8	32	13 (43.3)	0.5
Amikacin	2 - >256	256	>256	28 (93.3)	1
Gentamicin	4 - >256	64	256	28 (93.3)	0.5
Ciprofloxacin	8 - >32	>32	>32	30 (100)	0.5

MIC- minimum inhibitory concentration.

of the other carbapenem IMP- from 1 to >32 mg/l.

93.3 % of *P. aeruginosa* strains were R to both AMK (MICs ≥32mg/l) and GEN (MICs >8 mg/l) according to NCCLS-2003 (24). The most widespread mechanism of aminoglycoside resistance involves modification of the antibiotics by bacterial enzymes, plasmid or transposon encoded: acetyltransferases (AACs), adenylyltransferases (AADs) or phosphotransferases (APHs) (31). All studied strains were R to CIP, with MICs values ≥8 mg/l. The most important mechanism of quinolone resistance represents structural alterations of the primary (DNA gyrase) or the secondary (topoisomerase IV) targets of these antimicrobials because of chromosomal mutations respectively in gyrA/gyrB or in parC/parD genes (13).

The strains were screened for a presence of ESBL production by the DDST of Jarlier et al. (14). 14 strains of all 30 CAZ-resistant P. aeruginosa (46.7 %) were presumptive producers of ESBLs from molecular classes A or D (7) according to this phenotypic method. These strains also expressed synergism between IMP and CAZ suggesting PER-1, VEB- or GES-like enzyme production (34).

12 strains (40 %) were R to all beta-lactams, including to carbapenems. It was a phenotype of metallo-beta-lactamases (MBLs)-producing strains (16, 26). MBLs from molecular class B (7) are characterized with a very broad substrate profile, including expanded-spectrum cephalosporins (CAZ, FEP, CPO) and carbapenems (IMP, MER). Only the monobactams (ATM) are not hydrolyzed. The activity of these zinc-dependent enzymes is susceptible to EDTA, whereas it is not inhibited by clavulanic acid, tazobactam and sulbactam. The presence of MBLs was studied by the modified Hodge test (17). All strains showed a negative (-) Hodge test, therefore they were not the producers of MBLs and the resistance to beta-lactams resulted from the combination of different mechanisms such as: OprD deficiency, derepression of chromosomal AmpC cephalosporinase, ESBLs production and overexpression of active efflux systems.

The results from IEF and Bioassay together with the determined MIC values and results from DDST are presented in table 3. IEF analysis showed that 90 % of the strains contained 4 or 3 different beta-lactamases and 10 %- 2 beta-

Table 3. Presumptive beta-lactamases produced by the studied ceftazidime-resistant strains of Pseudomonas aeruginosa.

№	Year of isolation	Specimen	MICs (mg/l) CAZ	FEP	IMP	MER	DDSM (Jarlier)	IMP-CAZ DDSM	pIs	Bioassay CAZ, 2 mg/l	Presumptive beta-lactamases
1	2005	urine	>256	>32	1	0.19	+	+	5.7; 6.1; 7.4; 8.2	7.4+	CARB, OXA, VEB*, AmpC
2	2005	urine	>256	>32	>32	32	+	+	5.7; 6.1; 7.4; 8.2	7.4+	CARB, OXA, VEB, AmpC
3	2005	urine	>256	>32	1	0.25	+	+	5.7; 6.1; 7.4; 8.2	7.4+	CARB, OXA, VEB, AmpC
4	2005	urine	>256	>32	2	0.25	+	+	5.7; 6.1; 7.4; 8.2	7.4+	CARB, OXA, VEB, AmpC
5	2005	wound	>256	>32	1	0.19	+	+	6.1; 7.4; 8.2	7.4+	OXA, VEB, AmpC
6	2005	wound	>256	>32	1.50	0.25	+	+	6.1; 7.4; 8.2	7.4+	OXA, VEB, AmpC
7	2005	urine	>256	>32	2	0.75	+	+	5.7; 6.1; 7.4; 8.2	7.4+	CARB, OXA, VEB, AmpC
8	2002	wound	>256	>32	8	8	+	+	5.7; 6.1; 7.4; 8.2	7.4+	CARB, OXA, VEB, AmpC
9	2005	urine	>256	>32	1	0.19	+	+	5.7; 6.1; 7.4; 8.2	7.4+	CARB, OXA, VEB, AmpC
10	2001	BP	>256	>32	>32	32	-	-	5.7; 6.1; 7.4; 8.2	7.4+	CARB, OXA, VEB, AmpC
11	2002	wound	>256	>32	8	16	-	-	5.7; 6.1; 7.4; 8.2	7.4+	CARB, OXA, VEB, AmpC
12	2001	BP	>256	>32	2	1	+	+	5.7; 6.1; 7.4; 8.2	7.4+	CARB, OXA, VEB, AmpC
13	2002	wound	>256	>32	8	8	+	+	6.1; 7.4; 8.2	7.4+	OXA, VEB, AmpC
14	2002	BP	256	>32	16	16	+	+	5.7; 6.1; 7.4; 8.2	7.4+	CARB, OXA, VEB, AmpC
15	2002	urine	>256	>32	2	0.50	-	-	5.7; 6.1; 7.4; 8.2	7.4+	CARB, OXA, VEB, AmpC
16	2002	BP	256	>32	16	32	-	-	5.7; 6.1; 8.2	-	CARB, OXA, AmpC
17	2005	throat swab	>256	>32	32	16	-	-	5.7; 8.2	-	CARB, AmpC
18	2005	urine	>256	>32	8	8	+	+	5.7; 6.1; 7.4; 8.2	7.4+	CARB, OXA, VEB, AmpC
19	2002	urine	>256	>32	8	2	-	-	5.7; 6.1; 7.4; 8.2	7.4+	CARB, OXA, VEB, AmpC
20	2001	urine	32	>32	8	0.50	-	-	5.7; 8.2	-	CARB, AmpC
21	2004	urine	>256	>32	>32	>32	-	-	6.1; 7.4; 8.2	7.4+	OXA, VEB, AmpC
22	2001	nose swab	>256	>32	16	16	-	-	5.7; 6.1; 7.4; 8.2	7.4+	CARB, OXA, VEB, AmpC
23	2001	nose swab	>256	>32	32	16	-	-	5.7; 6.1; 7.4; 8.2	7.4+	CARB, OXA, VEB, AmpC
24	2001	BP	>256	>32	2	0.50	-	-	5.7; 6.1; 7.4; 8.2	7.4+	CARB, OXA, VEB, AmpC
25	2001	BP	>256	>32	16	16	-	-	5.7; 6.1; 7.4; 8.2	7.4+	CARB, OXA, VEB, AmpC
26	2001	BP	>256	>32	>32	16	-	-	5.7; 6.1; 8.2	-	CARB, OXA, AmpC
27	2001	wound	>256	>32	32	8	-	-	5.7; 6.1; 7.4; 8.2	7.4+	CARB, OXA, VEB, AmpC
28	2002	BP	128	>32	16	16	-	-	6.1; 8.2	-	OXA, AmpC
29	2004	rectal swab	>256	>32	>32	>32	-	-	6.1; 7.4; 8.2	7.4+	OXA, VEB, AmpC
30	2002	urine	>256	>32	16	8	+	+	6.1; 7.4; 8.2	7.4+	OXA, VEB, AmpC

BP- bronchopulmonary sample, MICs- minimum inhibitory concentrations, DDSM- double disk synergy method, pIs- isoelectric points, CAZ- ceftazidime, FEP- cefepime, IMP- imipenem, MER- meropenem; * VEB enzymes are extended-spectrum beta-lactamases.

lactamases. Nineteen strains (63.3 %) expressed several beta-lactamases with pI values of 5.7, 6.1, 7.4 and 8.2. Six strains (20 %) had beta-lactamase activities with pIs of 6.1, 7.4 and 8.2. Two strains (6.7 %) possessed 3 beta-lactamases focused at pIs of 5.7, 6.1 and 8.2. There were two strains of *P. aeruginosa* (6.7 %) produced 2 beta-lactamases, with pI values of 5.7 and 8.2. One of the studied strains (3.3 %) expressed 2 enzymes- pIs of 6.1 and 8.2.

The pI value of 5.7 and CAZ non-hydrolytic band (negative Bioassay test) likely corresponded to that of CARB enzymes from molecular class A and functional group 2c (7) such as PSE-1 (CARB-2) (pI of 5.7) and CARB-3 (pI of 5.75). The substrate profiles of PSE-1 and CARB-3 include carboxypenicillins, ureidopenicillins and cefsulodin but not ceftazidime and carbapenems (4). CARB producers are characterized with a variable susceptibility to cefepime, cefpirome and aztreonam (25). P. Nordmann reported a frequency of carbenicillin-hydrolyzing enzymes among clinical isolates of *P. aeruginosa* in France of 11 % and 90 % prevalence of PSE-1 producers (25).

The pI value of 6.1 and CAZ non-hydrolytic band (negative Bioassay test) probably corresponded to the narrow-spectrum OXA-10 enzyme from molecular class D and functional group 2d (7). Recently, Lee et al. (18) established that class D OXA beta-lactamases were more frequently detected than class A in P. aeruginosa from Korea (21 % versus 6.3 %), which contrasts with data from Europe (31.3 % versus 64.9 %) (4). OXA-10 was the most prevalent enzyme (13.5 %) in Korea (18).

25 strains from the total of 30 studied CAZ-resistant P. aeruginosa (83.3 %) showed CAZ hydrolytic bands with pI values of 7.4 (positive Bioassay) likely associated with the pI of VEB-1 ESBL from Ambler class A (12, 23). VEB-1 enzymes possess a wide substrate profile- hydrolyze carboxypenicillins, ureidopenicillins, extended-spectrum cephalosporins (CAZ, FEP, CPO) and aztreonam. They demonstrate a low affinity to carbapenems and their activities are inhibited by clavulanic acid and tazobactam (34). Only in 14 of the strains with pI value of 7.4 and CAZ hydrolytic bands, the DDST of Jarlier et al. (14) gave a presence of ESBLs and synergy between IMP and CAZ was observed. The double-disk synergy tests with clavulanate and extended-spectrum cephalosporins are sensitive and specific for the detection of ESBLs in Enterobacteriaceae (9). However, the same test may not be as useful for the detection of ESBLs in *P. aeruginosa* (34). Often false-negative results due to naturally occurring beta-lactamases, such as chromosome-encoded cephalosporinases (AmpC) that may be overexpressed (34). More over, the synergy between IMP and CAZ may be obscured in some cases by the induction effect of IMP on the expression of chromosomal AmpC beta-lactamase, resulting in a concomitant line of antagonism between CAZ- and IMP-containing disks (34). This effect can be overcome to some extend by performing the DDST with oxacillin-containing agar plates, since oxacillin inhibits the activity of Ambler class C enzymes (34). VEB-1-type ESBLs are widespread mostly in Asia (Thailand, India, China, Kuwait) (12, 23) and are rarely detected in European countries. Nevertheless VEB-1 appears to have a significant presence among nosocomial *P. aeruginosa* isolates in Bulgaria. Recently, Bachvarova et al. detected blaVEB-1 genes in 36.8 % of the investigated ceftazidime-resistant strains from more than 20 hospitals in distinct Bulgarian regions (1). blaVEB-1 are situated on class 1 integrons and are present as a gene cassette that varies in size and structure (33). In most cases, veb-1 cassette is associated with an oxa-10-like cassette encoding a narrow-spectrum oxacillinase-type beta-lactamase; with aminoglycoside resistance gene cassettes and an arr-2 cassette (rifampin resistance) (33). In our study all strains which expressed ESBL with pI value of 7.4 (likely VEB-1) contained also an other narrow-spectrum beta-lactamase focused at pI of 6.1 (probably OXA-10). These strains also were resistant to aminoglycosides- amikacin, gentamicin and tobramycin according to the NCCLS/2003.

Finally, all studied strains of P. aeruginosa expressed a beta-lactamase with pI of 8.2 likely corresponded to that of AmpC beta-lactamase (commonly called cephalosporinase) (12) from Ambler class C and functional 1 group (7, 27). AmpC is an inducible, chromosome-encoded enzyme. In the absence of beta-lactam antibiotics it is normally produced at low levels and confers resistance to aminopenicillins, first- and second-generation cephalosporins (27), but in the presence of inducing beta-lactams (especially imipenem) the expression may increase 100- to 1000-fold (2). The activity of AmpC is not inhibited by the currently available beta-lactam inhibitors.

In 5 from the total of 30 studied strains of *P. aeruginosa* (16.7 %) the resistance to expanded-spectrum cephalosporins, such as CAZ, FEP and CPO, was related to other mechanisms different from ESBLs-production: overexpression of a naturally produced AmpC cephalosporinase because of mutations in ampD; drug efflux or outer membrane impermeability (9, 27, 29).

CONCLUSION

The preliminary study of resistance mechanisms to beta-lactam antibiotics using methods, such as phenotypic detection of ESBLs, analytical IEF and Bioassay, established that the resistance to extended-spectrum cephalosporins was related to production of ESBLs, likely VEB-1, in 83.3 % of the investigated strains. VEB-1 is widespread mostly in Asia and is rarely in European countries. Nevertheless it appears to have a significant presence among CAZ-resistant *P. aeruginosa* isolates in Bulgarian hospitals and causes serious impediments in antimicrobial treatment. In the rest of ceftazidime-resistant strains (16.7 %) other mechanisms were implicated in resistance to third - and fourth-generation cephalosporins: overexpression of the chromosomally located AmpC beta-lactamase (stable derepressed cephalosporinase), increased efflux and decreased uptake by porin alteration. The carbapenem resistance observed in 40 % of P. aeruginosa strains was not related to enzymatic hydrolysis by a carbapenem-hydrolyzing metallo-beta-lactamase from molecular class B. The presumptive beta-lactamases produced by the studied strains of *P. aeruginosa* were: PSE-1 or CARB-3 from Ambler class A with pIs of 5.7-5.75- 76.7 %; narrow-spectrum OXA-10 from molecular class D (pI of 6.1) - 93.3 %; ESBL VEB-1 from Ambler class A (pI of 7.4) - 83.3 %; and AmpC cephalosporinase (pI of 8.2) among all strains. The obtained interesting results justify the necessity for additional molecular-genetic research such as PCR and DNA sequencing for exact detection and characterization of the enzymes.

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CHARACTERIZATION OF EXOCELLULAR NEURAMINIDASE FROM ERYSIPELOTHRIX RHUSIOPATHIAE

I. Abrashev

SUMMARY
A method for isolation and purification of neuraminidase from Erysipelothrix rhusiopathiae is described. Some of the characteristics of the enzyme as molecular mass, elecrophoretic mobility, isoelectric point, chemical nature etc. are presented.

Key words: Erysipelothrix rhusiopathiae, neuraminidase, molecular mass, isoelectric point, chemical nature.

INTRODUCTION

Neuraminidase (sialidase; N-acylneuraminisylgluco-hydrolase, EC 3.2.1.18) is an exo-glucosidase which is mostly found as terminal constituents of glycoproteins, glycolipids and olygosaccharides in higher animals and some microorganisms (1). Neuraminidase enzymes poses a key role in metabolism of sialoconjugates because of presence of sialic acids in biological molecules and cell membranes (2). Now-a-days many of the researchers use commercial neuraminidase products, which contain very often proteases and other enzymes and make difficult their biological, therapeutical and analytical application (3). In order to characterize neuraminidase activity by Erysipelothrix, many isolates of Erysipelothrix spp. From a variety of sources including human clinical, marine and terrestrial animals, and the environment were investigated for neuraminidase production (4). Neuraminidase from Erysipelothrix rhusiopathiae is not a commercial product up to now and that is why studies on its properties are difficult.

MATERIALS AND METHODS

MICROORGANISM AND GROWTH CONDITIONS. Erysipelothrix rhusiopathiae was inoculated in a medium on the basis of nutrient broth with the supplement of 0,03-0,06% glucose and 0,1-1% glucomacropeptide as inductor (5). The cultivation was done in 500 ml Erlenmeyer flasks with 100 ml of medium at 37°C for 30 hours. Cells were separated after 30 min. centrifugation at 10 000 rpm/min.

AMMONIUM SULFATE PRECIPITATION. The culture filtrate was mixed with $(NH_4)_2SO_4$ to 100% and after 24 hours precipitate formed was collected by centrifugation, dissolved in 0,1 M phosphate buffer, pH - 7 and dialyzed against tap water over night. The enzyme product was purified after that on ion-exchange chromatography, 50% precipitation with $(NH_4)_2SO_4$ and gel filtration on Sephadex G-200.

ASSAY OF NEURAMINIDASE ACTIVITY. Neuraminidase activity was established quantitatively according to Ushida (6). One unit of neuraminidase activity is defined as the amount that releases 1 xg of N-acetylneuraminic acid for 1 min. under standard condition using glucomacropeptide as a substrate (5).

ASSAY OF PROTEIN CONCENTRATION: Protein concentration was determined according to colorimetric method of Lowry et al. (7).

ASSAY OF HYALURONIDASE ACTIVITY: Hyaluronidase activity was established quantitatively according to Tolksdorf et al. (8).

ASSAY OF PROTEOLITIC ACTIVITY: Proteolytic activity was established quantitatively according to Matsubaro et al. (9).

ASSAY OF β-galactozidase: β-galactosidase activity was determined according to Kubi and Lardi (10).

OBTAINING OF ANTINEURAMINIDASE SERUM: The scheme of Schauer et al. (11) was used.

Results and discussion. Exo-cellular neuraminidase was purified according to modified method of Vertiev et al. (12) and the process comprises 4 stages: precipitation with

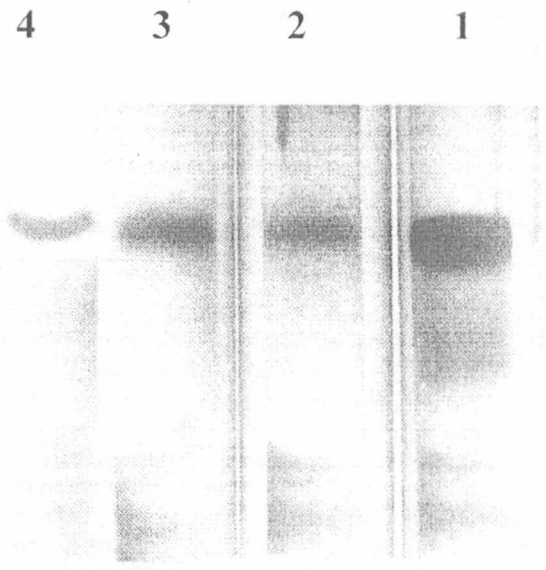

Fig. 1 Polyacrylamide gel electrophoresis
1. After precipitation with $(NH_4)_2SO_4$ - saturation 100%
2. After chromatography with DEAE-cellulose
3. After gel filtration with Sephadex G-200
4. After rechromatography with Sephadex G-200

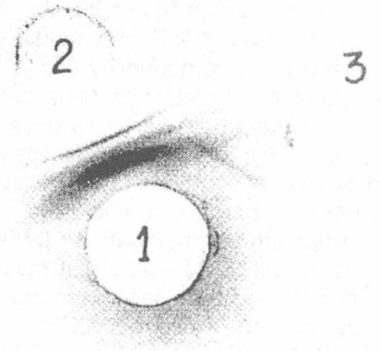

Fig. 2 Immunodifusion in agar gel
1. Antiserum against semi purified neuraminidase (after precipitation with $(NH_4)_2SO_4$ - 100% saturation)
2. Fraction after ion exchange chromatography with DEAE cellulose
3. Fraction after rechromatography on Sephadex G-200

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ABBREVIATIONS USED IN THIS PAPER: none

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Table 1. Purification of neuraminidase from *E. rhusiopathiae*

Purification step	Volume (ml)	Activity (U/ml)	Total Activity (U)	Protein (mg/ml)	Specific activity (U/mg protein)	Purification (-fold)	Yield (%)
Growth media	2000	49	98000				100
Precipitation with 100% $(NH_4)_2SO_4$	100	510	51000	20,1	25,4	1	52
Chromatography with DEAE	110	430	47300	1,7	252,9	9,95	48,2
Precipitation with 50% $(NH_4)_2SO_4$	11	4364	48004	3,0	1600	62,9	48,9
Gel filtration with Sephadex-G-200	6	7150	42900	3,5	2043	80,4	43,7

$(NH_4)_2SO_4$, ion-exchange chromatography on DEAE-cellulose and gel filtration on Sephadex G-200. All the results are presented on Table 1. After precipitation with $(NH_4)_2SO_4$, a 20-fold concentration of the initial material was achieved and after separation of the ballast nitrogen components - about 80%. The enzyme was 6-7 fold purified and the increase of the total activity was 10-30% compared to the cultural liquid. An essential stage of purification was enzyme chromatography on DEAE-cellulose. The specific activity of the isolated product was 250-300 U/mg protein. Precipitation with 50% $(NH_4)_2SO_4$ was used for concentrating of the received product. After this stage a great purification was observed - 60-65%. Next stage was gel filtration on Sephadex G-200.

Enzyme purification was 2000-fold compared to the initial preparation. Enzyme yield was about 40%. After rechromatography with Sephadex G-200 a pick of the enzyme was observed. PAGE and double immunodifusion in agar gel proved its homogeneity (Fig. 1, Fig. 2).

Polyacrylamide-gel electrophoresis gave as information both for enzyme purity and for its physiology characteristics - electric charge and molecular mass. Neuraminidase possesses strong cathode mobility, which was an evidence for its positive charge and acidic character. The enzyme from Erysipelothrix rhusiopathiae possesses molecular mass defined via SDS-PAGE about 350 000 D. High values of exo-cellular neuraminidase were found from Erysipelothrix rhusiopathiae - 345000-420000D (13), from Str. sanguis - 250 000 D (14). The results of analytical isoelectric focusing confirmed the data for acidic character of the enzyme. Isoelectric point of the enzyme is in the lower values of pH (pI=4,0). Biochemical assays did not show any other enzyme activities in the homogenous preparation (N-acetylneuramininatpyruvatelyase, hialuronidase, proteolytic and β-galactosidase activities). This way the preparation is suitable for biological investigations.

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CHARACTERIZATION OF AN EPIDEMIC METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS STRAIN SPREAD IN BULGARIAN HOSPITALS

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SUMMARY

The present study was designed to investigate the clonal relationship of methicillin-resistant Staphylococcus aureus (MRSA) strains isolated from different hospitals in Bulgaria. The isolates were analyzed by pulsed-field gel electrophoresis (PFGE) after SmaI digestion and by PCR amplification of the genes encoding coagulase (coa), the gene segments encoding X region and the IgG binding region of protein A (spa) and the genes encoding staphylococcal enterotoxin A (sea), B (seb), C (sec), D (sed), E (see), G (seg), H (seh), I (sei) and J (sej) and the toxic shock syndrome toxin 1 (tst). The isolates could be classified to PFGE profile type A with 2 subtypes, type B with 5 subtypes and to type C. All three types were considered to represent different MRSA strains. This classification could generally be confirmed by the indistinguishable PCR profiles of the genes spa and coa and by identical toxin gene patterns. The prevalent MRSA type A strain caused outbreaks in three hospitals in 1995, 1998 and 1999. The same strain could also be isolated from sporadic cases of infection and colonization in two other hospitals and from outpatient clinic. This PFGE type A MRSA strain was isolated from more than two patients and from more than two hospitals and could be defined as epidemic MRSA strain. The isolates belonging to PFGE type B caused an outbreak in one hospital and occurred sporadically in a second hospital. The third PFGE type C was found in sporadic cases in one hospital and in outpatient clinic. According to the data of the present study, a single epidemic MRSA strain seems to be responsible for infections of patients in at least five major Bulgarian hospitals.

Key words: Staphylococcus aureus; MRSA; clonal relationship; epidemic strain; PFGE; Bulgaria

INTRODUCTION

Methicillin-resistant Staphylococcus aureus (MRSA) emerged rapidly after the introduction of this antibiotic and soon has become an important clinical problem worldwide. The primary route of spread of MRSA was shown to be through clonal dissemination. Some MRSA strains, called epidemic MRSA, disseminated quickly and have been shown to spread within hospitals, between hospitals and between countries (1).

In the early 1980s, a single MRSA strain caused outbreaks in several hospitals in England and Wales (2). This strain was called epidemic MRSA-1 (EMRSA-1) to distinguish it from strains which do not cause outbreaks. Since then other epidemic strains have been described (EMRSA-2 through EMRSA-17) and EMRSA-15 and EMRSA-16 have been shown to be the dominant types of MRSA found in UK hospitals (3). The spread of a particular epidemic MRSA strain had also been described in Germany, Portugal, Poland, Hungary, the Czech Republic and in many other countries also including Greece and Turkey (3-9). Reports documenting the clonal spread of MRSA in Bulgaria are still limited in number and focused mainly on single hospital outbreaks and investigations with phenotypic methods (10). The aim of the present study was to investigate the clonal relationship of MRSA isolates from different hospitals in Bulgaria by molecular typing techniques.

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ABBREVIATIONS USED IN THIS PAPER: MRSA-Methicillin Resistant Staphylococcus Aureus
PFGE-Pulsed-field Gel Electrophoresis

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MATERIALS AND METHODS

A total of 54 clinical and colonizing MRSA from from six hospitals were included in this study. Twenty three isolates were from blood, 23 from wounds, 3 from respiratory specimens, 2 from urine and 14 from nasal swabs. The isolates were selected from strains sent for confirmation and typing to the Reference Laboratory for Staphylococci, National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria from 1995 to 1999. Thirty three isolates were collected from local outbreaks in hospitals I, III, V and VI. The additionally included 18 MRSA from hospitals II and IV were isolated from sporadic cases of infection and colonization. Only one isolate from a single patient was investigated. In addition, three ambulatory isolates collected from outpatients in Sofia were included in the study. The hospitals III, IV, V and VI are university teaching hospitals located in Sofia, western Bulgaria. The hospitals I (university hospital) and II (district hospital) are in northern and north-eastern Bulgaria and are located approximately 200 km apart from each other and about 180 and 360 km, respectively from Sofia.

Methicillin resistance was screened by oxacillin disk (1 (g, BD Microbiology systems, Cockeysville, USA), cefoxitin disk (30 (g, BD) and oxacillin-salt screen agar with 6 (g/ml oxacillin (BD) according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS)(11) and confirmed by PCR analysis of mecA gene, performed as described by Toshkova et al.(12).The isolates were further analyzed by pulsed-field gel electrophoresis (PFGE) after SmaI digestion and by PCR amplification of the polymorphic genes encoding coagulase (coa), the gene segments encoding X region and the IgG binding region of protein A (spa) and the genes encoding staphylococcal enterotoxin A (sea), B (seb), C (sec), D (sed), E (see), G (seg), H (seh), I (sei) and J (sej) and the toxic shock syndrome toxin 1 (tst). The preparation and digestion of genomic DNA for PFGE was performed as described (13) and the restriction patterns were interpreted according to Tenover et al.(14). The determination of the genes encoding coagulase (coa), the gene segments encoding X-region and IgG-binding region of protein A (spa), and the genes encoding staphylococcal toxins was performed as described previously (15). For control purposes the following S. aureus reference strains were used: 619/93 (sea), 62/92 (seb), 1229/93 (sec), 1644/93 (sed), FRI 918 (see), 161/93 (tst), Ly 990055 (seg, sei), Ly 990552 (seh). The strains were kindly provided by W. Witte, Robert-Koch-Insitut, Wernigerode, Germany and G. Lina, Centre Nationale des Toxemies a Staphylococques, Lyon, France. Positive controls for the remaining PCR reactions were obtained from the strain collections of the Institut fur Pharmakologie und Toxikologie and Staatliches Untersuchungsamt Hessen.

Table III. Origin, year of isolation and genotypic properties of the 54 MRSA isolates.

Setting	Year of isolation	n	PFGE pattern	coa	spa (X-region)	spa (IgG-binding region)	Toxin gene pattern
Hospital I	1998	9	A1	600[a]	124	540	sea, seh
		1	A1	600	124	700	sea, seh
Hospital II	1995	1	A1	600	124	540	sea, seh
	1996	2	B1	660	280	700	sea, sed, seg, sei, sej
		1	B1	660	300	700	sea, sed, seg, sei, sej
		1	B2	660	280	700	sea, sed, sej
		3	C	660	330	700	seg, sei
	1997	1	A1	600	124	540	sea, seh
		2	B1	660	280	700	sea, sed, seg, sei, sej
		1	B3	660	280	700	sea, sed, seg, sei, sej
		1	B4	660	280	700	sed, seg, sei, sej
		1	C	660	330	700	seg, sei
	1999	1	B1	660	280	700	sea, sed, seg, sei, sej
		1	B3	660	280	700	sea, sed, seg, sei, sej
Hospital III	1998	4	A1	600	124	540	sea, seh
Hospital IV	1998	1	A1	600	124	540	sea, seh
		1	A2	600	124	540	sea, seh
Hospital V	1995	15	A1	600	124	540	sea, seh
Hospital VI	1999	1	B1	660	280	700	sea, sed, seg, sei, sej
		3	B1	660	280	700	sed, seg, sei, sej
Ambulatory	1995	1	B5	660	280	700	sea, sed, seg, sei, sej
	1999	1	A1	600	124	540	sea, seh
	1999	1	C	850	300	700	seg, sei

n=number of isolates
[a] Approximate size in bp

RESULTS AND DISCUSSION

The DNA-fingerprinting by PFGE revealed that the 54 MRSA isolates could be distributed into the three PFGE types A, B and C. The predominant PFGE type A included the two subtypes A1 (n=33) and A2 (n=1), PFGE type B the five subtypes B1 (n=10), B2 (n=1), B3 (n=2), B4 (n=1) and B5 (n=1). All five isolates belonging to PFGE type C showed identical restriction patterns (Table 1).

The transmission of MRSA within and between the hospitals has been well documented using molecular typing techniques. A large molecular epidemiologic study of more than 3000 MRSA isolates from different regions of the world revealed the existence of only a few epidemic clones spread worldwide, namely the Iberian, Brazilian, Hungarian, New York/Japan, Paediatric and EMRSA-16 clone (1). One or more clonal types often predominate within the hospital settings in a given country. Oliveira et al.(16) reported that in the largest Portuguese teaching hospital, during three distinct surveillance periods, the Iberian and Brazilian clones were present in different proportions but together accounted for virtually all the MRSA isolates. De Lencastre et al.(6) reported about the existence of a unique epidemic MRSA clone, in both invasive and colonizing strains, which is widely dispersed in Hungarian hospitals hundreds of kilometers apart. In the Czech Republic, the Brazilian clone represented 80% of the MRSA isolates collected between 1996 and 1997 (7). Recently, Melter et al.(17) reported about the displacement of the Brazilian clone in the Czech Republic by a new clone, named Czech clone. Petinaki et al.(8), based on PFGE typing of MRSA isolates from five major hospitals in Greece, reported about the prevalence of three major clonal types.

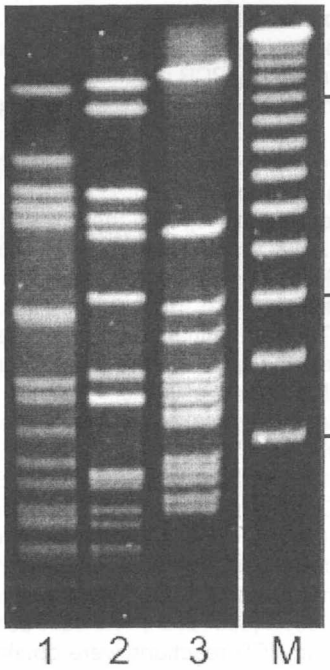

Figure 1. Typical PFGE patterns of SmaI-digested genomic DNA from MRSA isolates: lane 1, PFGE subtype A1; lane 2, PFGE subtype B1; lane 3, PFGE type C; M, lambda DNA concatemer standard

The S. aureus isolates of the present study were classified into three PFGE profiles, which were considered to represent different MRSA strains. The prevalent MRSA strain (PFGE type A) caused outbreaks in 1995, 1998 and 1999 in hospital I, III and V. The same strain was isolated from sporadic cases of infection and colonization in hospital II and IV and from ambulatory setting. As defined previously (3-9), since this MRSA strain was isolated from more than two patients and in more than two hospitals, it obviously could be defined as epidemic MRSA strain. The isolates belonging to PFGE type B were isolated from an outbreak in hospital VI and were also detected in hospital II in 1996, 1997 and 1999. This strain could possibly be classified as endemic for hospital II, since it persisted in this hospital during the period of four years. The third PFGE type C was found in sporadic cases in hospital II and for one ambulatory isolate.

The classification of the studied MRSA isolates to a particular clonal type could additionally be demonstrated by identical PCR profiles of the genes coa and spa. The determination of gene polymorphisms of coa and spa genes generally revealed an indistinguishable size of the amplicons in isolates assigned to the same PFGE type (Table 1). However, the differences in size of the coa gene and spa gene in three isolates of identical PFGE types could

be explained as ongoing evolution of the strain by deletion or insertion of segments in these regions. The variability in the genes coa and spa had been commonly used for epidemiological typing of *S. aureus* (18).

According to the results of the toxin genotyping, most of the isolates with indistinguishable or closely related PFGE type shared identical toxin gene patterns (Table 1). As described previously, genotyping *S. aureus* by determination of toxin patterns appears to be an useful technique for comparing epidemiologically related strains (19). In the present study, the prevalent MRSA strain (PFGE type A) was characterized by the presence of the staphylococcal enterotoxin A and H encoding genes sea and seh. The isolates assigned to PFGE type B were generally positive for the genes encoding SEA, SED, SEG, SEI and SEJ. However, some MRSA PFGE type B isolates did not show this toxin pattern. These differences could possibly be explained by a mutation of primer binding sites or a variation in the enterotoxin gene cluster. The isolates exhibiting the third PFGE type C were characterized by the presence of the genes encoding SEG and SEI. All of the seg positive isolates in this study were simultaneously positive for sei and all of the sed positive cultures were positive for sej. As shown previously, the genes encoding SEG and SEI are located in the enterotoxin gene cluster (egc)(20) and SED and SEJ are encoded by a plasmid and separated from each other by an intergenic region (21).

In conclusion, the epidemiological relation of the MRSA isolates investigated in the present study could be determined by SmaI restriction fragments analysis, by determination of gene polymorphisms of the genes coa and spa and by toxin genotyping. According to these results, a limited number of MRSA clones seem to be present in the hospitals investigated. An epidemic MRSA strain spread in at least five major Bulgarian hospitals could be defined.

Acknowledgments

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EXPRESSION LIBRARY IMMUNISATION REVEALING IMMUNO-DOMINANT DETERMINANTS OF FRANCISELLA TULARENSIS USING PLASMID DNA IMMUNIZATION TO SCREEN SUB-LIBRARIES FOR INDUCTION OF ANTI-FRANCISELLA TULARENSIS RESPONSES IN MICE.

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SUMMARY

The Expression-Library Immunization (ELI) technique was used to take advantage of the relatively small genomes of the pathogen F.tularensis and the already established protocols for plasmid DNA immunization in rodents. Two separate representative genomic libraries have been created and tested for that pathogen. The first -encodding secreted products to be processed via the class II MHC presentation pathway and stimulated CD4, CD8 T cell and humoral immunity. Alternatively, products from the second one targeted to the proteasome and processed via the class I MHC presentation pathway for primarily CD8 T cell stimulation. Immunization studies were conducted in a DBA mouse model and the expression libraries were screened for protection against F.tularensis. Mice were immunized with expression libraries containing the entire genome of the pathogen. Partial (prolongation of survival) protection from pathogen-induced disease in immunized animals identified sub-libraries that contain plasmids encoding for reactive antigenic epitopes. In this way, expression library immunization provides an unbiased, systemic approach for isolating vaccine candidates.

INTRODUCTION

Immune protection against *F.tularensis* involves both innate and adaptive immunity (1). The search for antigens, which are able to induce cellular responses, has not yet been successful. Future efforts to identify protective protein subunits might progress along three lines of investigations. First, classically, protective bacterial antigens are also considered to be virulence determinants and knowledge of virulence mechanisms might provide insight into likely vaccine candidates (2). Alternatively, a technique called "reverse vaccinology" has been applied to a range of other pathogens(3). This approach is based on the prediction of all open reading frames (ORFs) within the genome sequence for the identification of proteins that are likely vaccine candidates. We have selected the third approach, the Expression Library Immunization (ELI) technique (4), taking advantage of the relatively small genomes of Ft and the possibility to access the class I MHC presentation pathway of the host antigen presenting cells (5). Protocols for DNA immunization in rodents have already been established. Currently, there are no approved vaccines against Francisella tularensis (Ft).

In 1995, Barry et al. (6) published on a technique, termed expression library immunization (ELI) for identifying protective genes. This procedure capitalizes on the fact that a pathogenьs genomic DNA encodes all of the antigens produced by that pathogen. Hence, by immunizing with sequentially divided protective fractions of a genomic library, one has the potential of screening every gene in the pathogenьs genome. The strategy also offers the advantage of presenting the host with multiple genes, thereby simulating the effects of the live organism in the absence of any risk of infection.

Methods and reason of their application and results obtained:

Entire design:

Briefly, animals were immunized intramuscularly (i.m.) with the plasmid DNA isolated from respective sub-libraries and inoculated intraperitoneally (i.p.) 14 days later with the respective pathogen. Survival was monitored and compared with control mice that have been injected with PBS or with "empty" vector. We obtained partial protection identified that part of F.tularensis DNS containing fragments encoding immunodominant epitops.

Preparation of an active F.tularensis plasmid genomic libraries in two expression vectors directing the expressed proteins in either to the secretion pathway or proteasomal degradation The need for two types of libraries. It has been shown that immunization with expression library fragments that have been fused to a secretory gene sequence leads to augmented antibody response, whereas immunization with fragments inserted in-frame downstream of an ubiquitin sequence enhances cytotoxic lymphocyte responses (7). We have also found that deliberate inhibition of the translocation of proteins to the endoplasmic reticulum following transfection increases their proteasomal degradation and the generation of sub-dominant epitopes for MHC presentation {Mincheff, 2003}. We achieve this by either removing the signal peptide sequence of secreted or type I membrane proteins, or the transmembrane domain of type II membrane proteins. In this way the expressed products are not N-glycosylated but are retained in the cytoplasm and are targeted for proteasomal degradation to peptides {Mincheff, 2003}. Immunization with such constructs results in strong CD8 cytotoxic immunity and in no antibody formation against the native protein. Alternatively, CD4 T cell activation and antibody secretion is observed when immunization is performed with a DNA construct whose product is secreted (8) (Mincheff M. et al, manuscript in preparation).

Since in animal experiments both CD4 and, to a greater extent, CD8 T cells controlled the intracellular growth of Francisella tularensis (9), we have decided to create two separate expression libraries:

* The first, "secretable" library (SL) encoded secreted products that will be processed via the class II MHC presentation pathway and will primarily stimulate CD4 T cell and humoral immunity.
* Alternatively, products from the second, proteasome-targeted library (PL), targeted to the proteasome and processed via the class I MHC presentation pathway for CD8 T cell

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ABBREVIATIONS USED IN THIS PAPER: ELI-Expression Library Immunization

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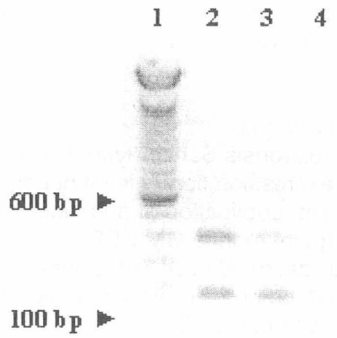

Fig.1 Multiplex PCR of SCHU S4 Ft DNA used for expression library construction.
1: Stratagen 100-bp ladder marker
2: SCHU S4 Ft DNA isolate
3: Sau3A-digested and size-fractioned SCHU S4 Ft DNA
4: no-template negative control.

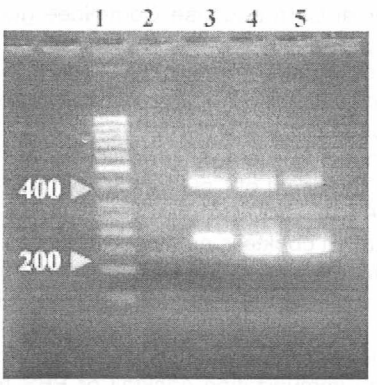

Fig.2 Comparative multiplex PCR of type A (SCHU S4) and type B Ft DNA
1: MW marker (50 bp ladder)
2: negative control (no template)
3: Ft SCHU S4 (type A)
4: Ft SCHU S4 and Ft subsp. holarctica (Srebarna 19 Bulgaria strain) mix
5: Ft subsp. holarctica (Srebarna 19 Bulgaria strain), type B

stimulation.

Library quality controls.

The standard quality controls are applied to each library. The following control transformations are performed:

1. Transformation with digested vectors - negative controls for restriction quality.
2. Transformation with digested self-ligated vectors - positive controls for restriction/ligation quality
3. Transformation with digested, dephosphorylated, self-ligated vectors - negative dephosphorylation controls.
4. Transformation with intact Ft DNA - negative control for the contamination of source DNA with foreign plasmid and/or for any ampicillin resistance associated with Ft DNA sample.
5. Transformation with Sau3A digested and gel purified Ft DNA with and without self-ligation - negative control for in-lab contamination.

Library diversity/authenticity assessment.

The DNA that was used for library construction was checked for Ft origin using a PCR-based species discrimination approach. The following primers were used:

Tul4-863: 5'-TTGGGAAGCTTGTATCATGGCACT-3'
Tul4-435: 5'-GCTGTATCATCATTTAATAAACTGCTG-3'
C4: 5'-GCGCGGATAATTTAAATTTCTCATA-3'
C1: 5'-TCCGGTTGGATAGGTGTTGGATT-3'

The first primer pair targets the conserved Tul4 gene which is present in all Ft species. The second primer pair targets a variable region in the Ft species.

Multiplex PCR performed with the primers showed the expected 400 bp band of the conserved Tul4 gene and a "lower-than-200" band specific for the Ft type A strains (fig.1). The SCHU S4 origin of the DNA was additionally verified by comparing it with DNA from a Ft type B (Srebarna Bulgaria strain) using the same primers (fig 2).

The band obtained with the primers targeting the variable region runs lower in Ft type B compared to type A strains, which additionally confirms the SCHU S4 origin of the DNA the library is constructed from.

For further diversity/authenticity assessment, the master library was plated to a single-colony density, and plasmids isolated from 54 randomly picked colonies were sequenced using vector-specific primer 5'-CAGGAGAGGCACTGGGGAGGGTCAC-3'. The primer has no specificity to Ft DNA as checked by searching of false priming sites in Ft sequence database.

The obtained sequences were BLASTed against SCHU S4 and LVS databases available from http://artedi.ebc.uu.se/Projects/Francisella and http://bbrp.llnl.gov/bbrp/html/microbe.html respectively.

The sequence analysis shows that all non-empty clones contain Ft specific inserts. No foreign DNA was detected. Each clone represents an unique Ft sequence and no duplication or multiplication is found among sequenced clones. Three clones having SCHU S4 specific inserts were found to have no homology with LVS sequences and that gave additional support to SCHU S4 authenticity of the library.

Seven of the 54 clones (13%) contained no or short (<100 bp) inserts. The average insert length among the remaining 47 clones was 450 bp. The insert length frequency distribution histogram is present on fig.3. Empty clones as well as those having the inserts shorter than 100 bp were excluded from calculations.

Three of the 47 studied clones (6%) were found to have chimerical insert composed of DNA fragments belonging to different contigs in both SCHU S4 and LVS databases. These clones result from concatemerisation of DNA fragments.

Alignment of the analyzed clones with the SCHU S4 and the LVS genomes (fig. 4) shows no clustering proving that library evenly cover the genome. Sequences and/or trace files are available upon request.

Mice Immunization. Six- to 8-week-old, specific-pathogen-free DBA mice were housed in the BSL-3 Animal Core Facility at the Bulgarian National Center of Infectious and Parasitic Diseases in Sofia. Mice were fed autoclaved food and water ad libitum. All experiments were performed un-

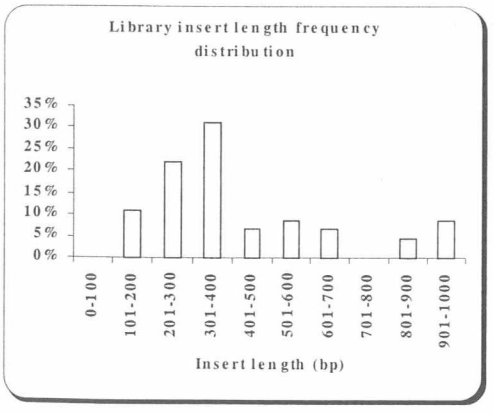

Fig.3. Library insert length frequencies distribution.

der the Animal Care and Use Committee guidelines and an approved protocol.

Three groups of mice (5 animals per group) were immunized with plasmid pools isolated from three sub-libraries (1000 clones each) as described above. Control animals were immunized with "empty" plasmid/GM-CSF cocktail to account for the effect of CpG motifs from vector DNA and the GM-CSF (10).

All immunization cocktails contained 1000 (g plasmid DNA and recombinant murine GM-CSF (9 mg/m2 body surface area). The dose of the recombinant GM-CSF has been determined from previous studies in rats, mice and humans (11) (M. Mincheff et al. unpublished observations). A total of 0.3 ml of the immunization cocktail or PBS (control PBS group) was injected i.m. per mouse.

All immunizations were performed by a standard intramuscular injection technique. The animals were observed once daily for clinical signs of toxicity (cage side). A more thorough examination was performed at weekly intervals. Animals were monitored for weight on weekly intervals throughout the study.

Francisella tularensis subsp. holarctica type B (strain Srebarna 19) was used to challenge the immunized and control mice. The strain was isolated in Bulgaria in 1962 (12) and is pathogenic to both humans and mice.

Francisella tularensis inoculation. *F. tularensis* subtype B (strain Srebarna 19) was cultured on a modified Mueller-Hinton (MH) agar plate (Difco Laboratories, Detroit, MI) in a humidified 37°C incubator with 5% CO2 or in modified MH broth (Difco Laboratories, Detroit, MI) in a 37°C air shaker. Stock cultures were grown overnight in broth from a single isolated colony to the stationary phase and frozen in broth in aliquots at -80°C. Viable c.f.u. after freezing and thawing were determined by plate counts of serial dilutions.

In preliminary tests, the LD50 for F. tularensis subtype B, strain Srebarna 19 in mice was determined to be 1-100 bacteria when infection was initiated by intraperitoneal (i.p.) inoculation.

Two weeks after the immunization, the immunized and the control mice were injected i.p. with 300 c.f.u. of Ft, subspecies B, strain Srebarna 19, resuspended in 0.5 ml of PBS, a dose that killed in 96 hours 100% of naive animals. Actual challenge doses of bacteria were confirmed by plate count at the time of inoculation. Mice were monitored daily for clinical signs of tularemia and survival. Survival of control and immunized animals was compared (fig.4). Protection in sub-library D is a basis for a consecutive subdivision of that part of bacterial DNA.

Conclusions:

Our studies showed that:

1. A Francisella tularensis Schu4 (type A) proteasome-targeted genomic expression library is immunogenic in mice
2. Mice tolerate i.m. application of a cocktail of 1 mg plasmid DNA and 9 (g/m² murine GM-CSF
3. A single immunization of mice with a sub-library having a complexity of 1000 members prolongs survival of mice after lethal challenge with Ft type B
4. Protection of mice after immunization with type A Ft genomic library can be tested after challenge with Ft type B.
5. Primary screening of library with a complexity of 1000 members can be performed after single immunization with 1 mg of total plasmid mass.

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