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PROBLEMS OF INFECTIOUS AND PARASITIC DISEASES
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ANTIMICROBIAL ACTIVITY OF DAPTOMYCIN - A NEW ANTIBIOTIC TESTED AGAINST METHICILLIN-RESISTANT STAPHYLOCOCCI AND VANCOMYCIN-RESISTANT ENTEROCOCCI ISOLATED IN DIFFERENT BULGARIAN MEDICAL CENTERS

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SUMMARY

Daptomycin is a cyclic lipopeptide with potent activity and broad spectrum against Gram-positive bacteria currently used for the treatment of complicated skin and skin structure infections and bacteremia, including right sided endocarditis. We evaluated the in vitro activity of this compound against clinical strains of staphylococci and enterococci collected from Bulgarian medical centers in the National Reference Laboratory for „Control and Monitoring of Antimicrobial Resistance" at the NCIPD.

A total of 100 non-duplicate clinical strains from different medical centers were tested for susceptibility by reference agar microdilution methods according to Clinical and Laboratory Standards Institute guidelines and interpretative criteria.

All S. aureus strains were inhibited at a daptomycin MIC of ≤1 mg/L. Among tested E. faecium strains the highest daptomycin MIC value was 2 mg/L (MIC50 under 0.5 mg/L), while among E. faecalis and E. avium the highest MIC value was 1 mg/L.

Daptomycin showed excellent in vitro activity against staphylococci and enterococci collected in the National Reference Laboratory for „Control and Monitoring of Antimicrobial Resistance" and appears to be an excellent therapeutic option for serious infections caused by methicillin-resistant staphylococci and vancomycin-resistant enterococci.

Key words: Daptomycin, MIC, serious infections, MRSA, VRE

INTRODUCTION

Gram-positive bacteria, especially staphylococci and enterococci, are extremely important pathogens causing severe infections mostly in the hospital environment. Staphylococcus aureus, coagulase-negative staphylococci (CoNS) and enterococci are among the five most frequently isolated organisms from nosocomial bloodstream infections (BSI) [1, 2].

These three pathogens are responsible for approximately one-half of the cases of BSI in North American medical centers evaluated by the SENTRY Antimicrobial Surveillance Program [3].

Over the last decade, methicillin-resistant Staphylococcus aureus (MRSA) strains have emerged as serious pathogens. These strains are often multiresistant to several antibiotic classes and are a major cause of serious hospital- and now community-acquired infections and associated morbidity and mortality. As a result of increasing antimicrobial resistance, glycopeptides, such as vancomycin, are widely used as first-line therapy for serious MRSA infections. However, the emergence of glycopeptide tolerance and resistance has complicated treatment and there remains a clinical need for new antibiotics with suitable pharmacokinetic properties with activity against MRSA and other Gram-positive pathogens. Infections caused by MRSA and other bacteria usually respond as well to bacteriostatic agents as to bactericidal ones. Nevertheless, there is evidence that rapid bacterial killing has potential clinical advantages over bacteriostatic therapy in certain infections.

Resistance to β-lactams and other agents has resulted in the increasing use of glycopeptides (i.e. vancomycin and teicoplanin), as first-line therapy for the treatment of serious MRSA infections. However, various forms of glycopeptide resistance have appeared in MRSA strains, including rare high-level resistance, homogeneous and heterogeneous intermediate resistance and glycopeptide tolerance. In response to this challenge, a number of new antimicrobials have been developed including the streptogramins (quinupristin/dalfopristin), the oxazolidinones (linezolid) and, more recently, the cyclic lipopeptides (daptomycin).

Daptomycin, the first of the cyclic lipopeptides, shows rapid bactericidal activity against S.aureus, including strains tolerant or resistant to other agents [4].

S. aureus isolates are usually inhibited in vitro by vancomycin concentrations of 0.5-2 mg/l. Isolates with vancomycin MICs of 8-16 mg/l are referred to as vancomycin-intermediate S. aureus (VISA), and those with vancomycin MICs of 32 mg/l are designated vancomycin-resistant (VRSA)[9]. Vancomycin-resistant or -intermediate isolates usually show similarly reduced susceptibilities to teicoplanin and may be referred to as glycopeptide-resistant or glycopeptide-intermediate S. aureus (GRSA or GISA).

Fully vancomycin- and teicoplanin-resistant S. aureus isolates are rare: only four such isolates (all methicillin-resistant) have been reported - all from the USA - between 2002 and 2005[3]. High-level resistance is encoded in these strains by the vanA transposon, probably acquired from vancomycin-resistant enterococci (VRE).

A dramatic rise in frequency of enterococcal infections and the prevalence of vancomycin-resistant enterococci (VRE) occurred during the 1990s in the USA, first in ICUs, then essentially throughout hospitals [5]. The 2004 NNIS (National Nosocomial Infections Surveillance) report indicated that nearly 30% of all enterococci isolated from patients infected in ICUs were resistant to vancomycin [2]. Although most European countries were able to control the hospital dissemination of VRE in the 1990s, the prevalence of this pathogen has recently increased dramatically in many European countries.

According to Christidou and Metallidis VRE carriage among patients in Greek University hospitals ranges between 20.5-30.5%. Nosocomial infection with VRE in Turkey was reported in 2006.

Daptomycin was originally isolated as a fermentation product of the soil-dwelling bacterium Streptomyces roseosporus. Daptomycin is a 13-amino-acid cyclic lipopeptide (1620 Da) comprising a hydrophilic core and a lipophilic tail. This unique

ABBREVIATIONS USED IN THIS PAPER:

BSI - BloodStream Infection, CLSI - Clinical and Laboratory Standards Institute, CoNS - Coagulase-Negative Staphylococci, cSSTI - complicated Skin and Soft Tissue Infection, EMEA - European Medicines Agency Testing, EUCAST - European Committee for Antimicrobial Susceptibility Testing, FDA - Federal Drug Administration, ICU - Intensive Care Unit, MDR - MultiDrug-Resistant, MIC - Minimum Inhibitory Concentration, MRSA - Methicillin-Resistant Staphylococcus aureus, MSSA - Methicillin-Susceptible Staphylococcus aureus, NCCLS - National Committee for Clinical Laboratory Standards, NNIS - National Nosocomial Infections Surveillance, VISA - Vancomycin-Intermediate Staphylococcus aureus, VRSA - Vancomycin-Resistant Staphylococcus aureus, VRE - Vancomycin-Resistant enterococci

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structure confers upon Daptomycin a completely novel mode of action involving insertion into the cytoplasmic membrane of Gram positive bacteria and the formation of Daptomycin pores. This Ca2+-dependent process leads to efflux of cell components, particularly potassium ions, and subsequent membrane depolarisation and cell death. Daptomycin kills bacteria with negligible cell lysis, so the theoretical risks associated with bacterial cell lysis - including the release of bacterial exotoxins into the circulation - are potentially minimised [7].

Glycopeptides inhibit peptidoglycan synthesis in the bacterial cell wall by complexing with the D-alanyl-D-alanine portion of the cell wall precursor. Daptomycin also acts by inhibiting bacterial peptidoglycan synthesis, but this is probably secondary to its interference with membrane transport of precursors. Resistance to glycopeptides is due to the presence of a complex series of bacterial cytoplasmic enzymes synthesizing abnormal peptidoglycan precursors terminating in D-ala-D-lactate, thereby markedly lowering the binding affinity with the glycopeptides.

Daptomycin has rapid bactericidal activity against Gram-positive pathogens. It acts by penetrating into the bacterial cell wall in the presence of physiological levels of calcium ions (50 mg/l) with consecutive formation of pores, loss of electrical membrane potential and inhibition of peptidoglycan synthesis. As the mode of action of Daptomycin is „concentration-dependent", the pharmacokinetic/pharmacodynamic indices that correlate best with its activity are the ratios of the peak concentration (Cmax) to minimum inhibitory concentration (MIC) or the area under the curve (24-hour AUC) to MIC. Intravenous infusion of Daptomycin at a dosage of 6 mg/kg results in peak and trough concentrations of 82 and 6 μg/ml, respectively, in serum. About 90% of the drug is bound to plasma protein, with limited metabolism. The elimination half-life is 9 h, and 80% of the drug is excreted via the kidneys, two-thirds as intact drug.

We evaluated the in vitro activity of this compound against clinical strains of staphylococci and enterococci collected during the last ten years in the National Reference Laboratory for „Control and Monitoring of Antimicrobial Resistance" at the NCIPD.

METHODS

Bacterial strains

A total of 100 contemporary clinical isolates, including 60 Staphylococcus aureus (MRSA) and 40 Enterococcus spp. (20 Enterococcus faecium, 15 E.faecalis and 5 E. avium) were evaluated in the present study. All isolates were non-duplicate, consecutive, clinical strains collected from patients hospitalized in different medical centers located in Bulgaria.

Identifications were performed by) using standard biochemical algorithms: Api® 20 Strep and Api® Staph (bioMerieux® SA) .

Antimicrobial agents and susceptibility testing

Daptomycin was tested by Clinical and Laboratory Standards Institute (CLSI) criteria [20]. All strains were tested for antimicrobial susceptibility by the broth microdilution method.

Our study was made according to Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobicalli; Approved Standard - Fifth Edition, M7-A7, CLSI, Vol.26 No.2, January 2006 and Performance Standards for Antimicrobial Suseptibility Testing; Seventeenth Informational Supplemen, M100-S17 (M7), CLSI, January 2007 .

Dry-form Daptomycin, manufactured by Hospira Inc., Kansas, USA for Novartis Pharma AG, Basel, Switzerland.

RESULTS

Gram-positive bacteria, especially staphylococci and enterococci, are extremely important pathogens causing infections in the hospital environment. Staphylococcus aureus, coagulase-negative staphylococci (CoNS) and enterococci are among the five most frequently isolated organisms from nosocomial bloodstream infections (BSI) [1, 2]. These three pathogens are responsible for approximately one-half of the cases of BSI in North American medical centers evaluated by the SENTRY Antimicrobial Surveillance Program [1]. National data on bloodstream infections (Fig. 1) from the Bulgarian Surveillance Tracking Antimicrobial Resistance for 2006 show similar results: Staphylococcus aureus is the leading pathogen (20.0%), Enterococcus spp. are third (11.3%), and CoNS are forth - with (9.7%) [28]. Regarding the SSIs data from BulSTAR show almost the same picture (Fig. 2). The role of Staphylococcus aureus and Enterococcus faecalis as hospital pathogens is increasing through the last years of BulSTAR Surveillance [29].

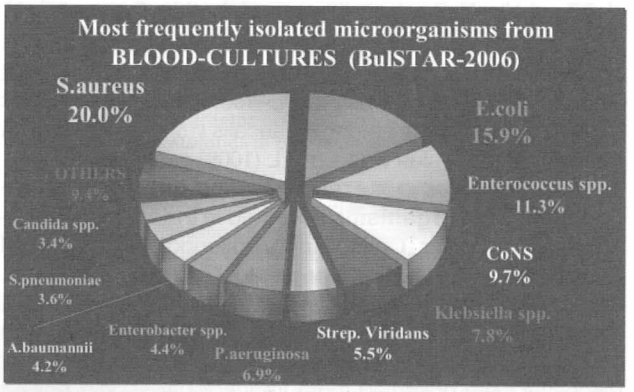

Fig. 1.

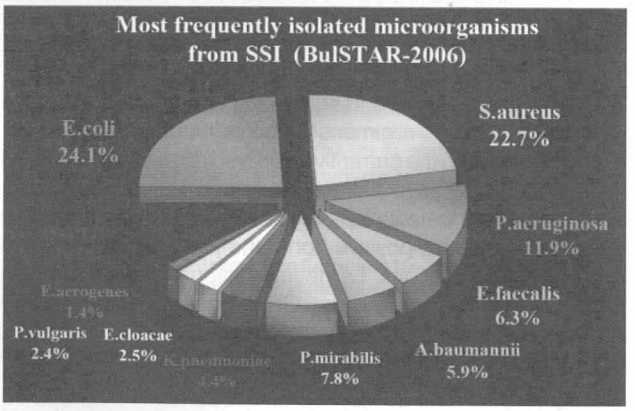

Fig. 2.

The isolates were categorized as susceptible and resistant according to CLSI guidelines [20]. A daptomycin susceptible breakpoint of ≤ 1 mg/L was used for staphylococci, while ≤ 4 mg/L was used for enterococci, as approved by the USA-FDA, CLSI and EUCAST [21,22].

Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobicalli; Approved Standard - Fifth Edition, M7-A7, CLSI, Vol.26 No.2, January 2006 and Performance Standards for Antimicrobial Suseptibility Testing; Seventeenth Informational Supplemen, M100-S17 (M7), CLSI, January 2007 .The following quality control organisms were concurrently tested: E.faecalis ATCC 29212, S.aureus ATCC 25923, E.coli ATCC 25922, P.aeruginosa ATCC 27853 and S.pneumoniae ATCC 49619.

Results Daptomycin:
MIC value for S.aureus (all confirmed as MRSA) - 0,125 - 1 μg/ml.
Results Daptomycin:
MIC value for E.faecium - 0,25 - 2 μg/ml
MIC value for E.faecalis - 0,25 - 1 μg/ml
MIC value for E. avium - 1 μg/ml

Table 1. Antimicrobial activity of daptomycin tested against Bulgarian S. aureus and enterococcal isolates

Strain/ Concentration (μg/ml)	0.1	0.3	0.5	1	2	4	8	16	32	64	128	256	512	Positive control (without antibiotic)
Enterococcus spp. (40 strains)														
Enterococcus faecium (20 strains)		4	5	1	10									20
Enterococcus faecalis (15 strains)		1	2	12										15
Enterococcus avium (5 strains)				5										5
MRSA (60 strains)		9	31	18	2									60

All S. aureus strains were inhibited at a Daptomycin MIC of ≤1 mg/L (100.0% susceptible). Daptomycin was also highly active against enterococci. Among 20 tested E. faecium the highest Daptomycin MIC value was 2 mg/L - 100% susceptible (MIC50 under 0.5 mg/L), while among E. faecalis (15) and E. avium (5) the highest MIC value was 1 mg/L (100% susceptible).

Daptomycin was generally very potent against the Gram positive organisms collected in the National Reference Laboratory for „Control and Monitoring of Antimicrobial Resistance"(the results are present in Table 1).

Daptomycin should be administered intravenously once daily, because adverse effects on skeletal muscle associated with an increase in plasma levels of creatine phosphokinase and myopathy were observed more frequently at shorter dosing intervals. Overall, the rate of adverse events during daptomycin therapy is comparable to that of other standard regimens. Daptomycin was shown to be not inferior to antimicrobial standard therapy and therefore was approved for complicated skin and skin structure infections at a dose of 4 mg/kg, for Staphylococcus aureus bacteremia and right-sided endocarditis at a dose of 6 mg/kg. Dosage regimens remain a matter of discussion, and an increase in the currently approved doses from 4-6 to 6-8 mg/kg per day for severe infections seems promising. Though not approved up to now, daptomycin appears to be a treatment alternative for Gram-positive bone and joint infections based on clinical observations. Large international studies showed high susceptibility of relevant Gram-positive pathogens to daptomycin, even in multidrug-resistant strains. Thus, treatment of infections caused by Gram-positive cocci resistant to other antimicrobial drugs is a potential indication of daptomycin. Since glycopeptides and daptomycin have the same target site, there appears to be a risk of reduced susceptibility to both drugs after consecutive use. Therefore, daptomycin should be used with caution for treatment of vancomycin-resistant isolates or after prior vancomycin (glycopeptide) therapy.

DISCUSSION

The treatment of serious MRSA infections presents a great challenge to clinicians, particularly bacteremias and infective endocarditis, for which bactericidal therapy is essential to maximize successful clinical outcomes [18]. Vancomycin has been the preferred antimicrobial agent to treat such MRSA infections; however, the clinical efficacy of this glycopeptide has become more limited [19]. In addition to the increasing reports of isolates with reduced susceptibility (vancomycin-intermediate S. aureus [VISA]) or high-level vancomycin resistance (vancomycin-resistant S. aureus [VRSA]), other reports have shown limited bactericidal activity against a large proportion of strains with vancomycin MIC values within the CLSI susceptible range.

Although they are relatively nonvirulent organisms, the enterococci have become increasingly common nosocomial pathogens because they are resistant to many antimicrobials and can survive in the environment for prolonged periods of time. Enterococci are intrinsically resistant to multiple antimicrobial agents. Furthermore, other agents that are active in vitro, such as vancomycin, are not bactericidal at clinically achievable concentrations. Combination therapy of a cell-wall active agent plus an aminoglycoside has become the „standard of care" for patients with serious enterococcal infections, such as endocarditis or BSI, but the prevalence of high-level resistance to aminoglycosides and to ampicillin are increasing, leaving glycopeptides as the remaining class of active antimicrobials. Clearly, the emergence of VRE has further complicated therapeutic options [27].

Two antimicrobial agents have been approved specifically for the treatment of VRE infections: a streptogramin combination quinupristin/dalfopristin and an oxazolidinone linezolid [25]. However, quinupristin/dalfopristin MIC90 results (16 mg/l) for E. faecalis systemic infections exceeds the maximum achievable serum concentrations, making this compound inactive for E. faecalis, and resistance among E. faecium has been recently increasing, especially in Europe among vancomycin resistant strains [27]. On the other hand, linezolid has potent in vitro activity against both vancomycin-resistant E. faecalis and E. faecium, as well as good therapeutic efficacy for VRE bacteremia in mice [25]. However, linezolid has not been recommended for the treatment of endocarditis or serious infections in immuno-suppressed patients due to its predominantly bacteriostatic activity. In addition, the emergence of oxazolidinone resistance has been reported, especially in patients who receive prolonged courses of therapy.

Linezolid and quinupristin-dalfopristin represent alternative treatment options for serious MRSA infections; however, these compounds also possess important limitations. Quinupristin-dalfopristin requires a central venous access to be administrated and has been linked to some adverse events such as arthralgia and myalgia [25,26]. Concerns with linezolid include possible hematologic toxicity of long-term treatment and the fact that it is a bacteriostatic agent against staphylococci and enterococci and is not indicated for the treatment endocarditis and serious infections in immuno-suppressed patients.

Daptomycin is the first member of a novel class of antimicrobial agents, the cyclic lipopeptides [8]. It has broad-spectrum and potent bactericidal activity against Gram-positive pathogens, including MRSA and VRE [10]. This compound has demonstrated activity against both growing and stationary-phase bacteria [4]. Here Daptomycin was recognized as highly active against S. aureus and CoNS, all enterococci, including vancomycin-resistant strains were susceptible to Daptomycin.

Daptomycin was approved by the USA Food and Drug Administration (FDA) in September 2003 for the treatment of complicated skin and skin structure infections (cSSTI) at a dosage of 4 mg/kg every 24 hours; and more recently for the treatment of bacteremia with or without right sided infectious endocarditis at a dosage of 6 mg/kg every 24 hours

[21, 23]. Daptomycin is not indicated for the treatment of community-acquired pneumonia (CAP). The lack of efficacy of daptomycin in CAP is thought to be due to a reduction of daptomycin activity in the presence of lung surfactant. Daptomycin has also been recently approved by the European Medicines Agency (EMEA) for the treatment of cSSTI [22]. In addition, the European Committee for Antimicrobial Susceptibility Testing (EUCAST) has assigned Daptomycin breakpoints for staphylococci and streptococci [24], which are ≤1 mg/L for susceptible (similar to Clinical and Laboratory Standards Institute [CLSI] and US-FDA breakpoints) and ≥2 mg/L for resistance [20].

In contrast to other classes of bactericidal antibiotics, the rapid bactericidal activity of Daptomycin does not require cell division or active metabolism, and Daptomycin retains bactericidal activity against non-growing S.aureus cells under a variety of physiological conditions.

Extensive testing has shown that Daptomycin possesses rapid bactericidal activity which is maintained against both high inocula and non-growing bacterial cells. Daptomycin has a prolonged PAE and shows synergy with certain other antibiotic agents. The potential to induce Daptomycin-resistant mutants in vitro is low, and resistant isolates have only rarely been recovered from Daptomycin-treated patients. Interesting areas for future testing include further characterisation of Daptomycin's mode of action, its protein binding and its activity in biofilms. It is already clear, however, that Daptomycin represents an exciting new option for the treatment of Gram positive infections.

CONCLUSIONS

The general conclusions of our survey are: the antimicrobial activity of Daptomycin against Gram-positive bacteria, including those with problematic resistance like MRSA, ampicillin-resistant E.faecium, High Level Aminoglycoside Resistant Enterococci, is very good, compared to similar antibiotic agents. All tested strains were inhibited by Daptomycin MICs significantly lower than the corresponding breakpoints according to the CLSI for 2008. During this National study no resistant bacteria were found in Bulgaria and 100 % of the tested strains were susceptible to Daptomycin.

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IMMUNE RECOVERY OF HIV+ PATIENTS WITH DIFFERENT BASELINE CD4 ABSOLUTE COUNTS AFTER 24 MONTHS OF HIGHLY ACTIVE ANTIRETROVIRAL THERAPY

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SUMMARY

Human immunodeficiency virus (HIV) infection leads almost inevitably to progressing immune deficiency. Timely administration of highly active antiretroviral therapy (HAART) decreases viral load (VL) and permits the preservation and partial restoration of the CD4 T cell pool. Although CD4 absolute counts (CD4 AC) between 200 and 350 cells/μl are accepted as a paramount criterion for starting HAART, choosing the right time has proved a complicated issue. In order to set variables predictive for an effective response to therapy, we monitored the changes in CD4AC, VL and CD38 quantitative expression (CD38 ABC) on CD8 T cells in the course of 24 months of HAART in treatment-nanve HIV+ patients (average CD4 AC 115 cells/μl, range 2 - 431 cells/μl), sub grouped according to baseline CD4 counts. At the endpoint, patients were retrospectively classified according to the effect of HAART into: responders (sustained immunologic and virologic response), transient responders (transient/absent immunologic and virologic response) and paradox responders (immunologic response combined with ongoing HIV replication). According to our results, the effect of HAART was independent from baseline CD4 counts. Our data provide strong evidence that baseline CD4 cell count alone cannot predict the effect of therapy in patients with advanced HIV infection and CD4 AC < 400 cells/μl. Considering other parameters such as CD38ABC might be helpful.

Key words: HIV-1 infection, HAART, immune recovery, CD4 absolute count, CD38

INTRODUCTION

At present, highly active antiretroviral therapy (HAART) is the only efficient, though not definitive means to fight HIV infection (2). The possibility to reduce viral load to an undetectable level prevents or significantly slows down immune destruction. On the other hand, HAART is a life-long therapy, with important side effects and compliance proves difficult on the long run. Therefore, defining the right moment and criteria to start antiretroviral treatment is crucial for the patients. Large retrospective studies have been undertaken to clarify the most important predictive variables that would help choosing the right moment to start HAART. The value of CD4 absolute count (350 cells/μl) has been so far the major variable considered for starting HAART and monitoring and /or evaluating its course (16;24). On the other hand, therapy effect has been evaluated rather as the complex of increasing CD4 AC and decreasing viral load, since the recognition of paradoxical effect of therapy (increasing CD4 AC in spite of important viral load) and vice versa (7;22;33).

Recently, some studies have put into question the significance of baseline CD4AC itself as a predictive factor of therapy effect (25).

In order to further elucidate this issue, we analyzed the two basic determinants of immune recovery: CD4AC and viral load in the course of 24 months of HAART in patients with low, medium and high baseline CD4 counts.

ABBREVIATIONS USED IN THIS PAPER:
HIV - human immunodeficiency virus, HAART - highly active antiretroviral therapy

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MATERIALS AND METHODS

Patients. The study included: 55 treatment-nanve HIV-1+ patients from the Hospital of Infectious Diseases, Sofia (male/female ratio: 36 male, 19 female, median age 36.7), receiving HAART (2 NRTI and 1 protease inhibitor) in the course of 24 months. Heparinized peripheral blood samples were obtained after informed consent in the course of routine monitoring of CD4 absolute count (AC). Samples were taken at baseline (the first day of HAART administration) and every 3 months thereafter until the endpoint (24 months of HAART).

Immunophenotyping and flow cytometry. Blood samples for phenotyping and quantification of CD4 T cells were collected in heparinized vacutainer (B-D) tubes and stained within 4 hours. Multicolor colour immunophenotyping on whole blood was performed as previously described (31). Peripheral blood CD4+ T cell absolute counts (CD4 AC) were determined by the CD3/CD4/CD8 TriTEST with TruCOUNT tubes (B-D) by a lysis/no wash procedure using a FACSCalibur flow cytometer and CellQuest software and further analysed by MultiSET and Attractors software. Immune activation was assessed according to the quantitative expression of CD38 (CD38 antibody binding capacity, ABC) on CD8 T cells using the QUANTIBRITE PE fluorescence quantitation kit and, QUANTIBRITE software according to manufacturer's instructions (BD Biosciences, San Jose, CA) and analyzed by QUANTIBRITE software.

Viral load determination. HIV-1 RNA plasma levels were determined on EDTA-anticoagulated plasma by RT-PCR using the Aplicor HIV-1 Monitor v.1.5 (Roche Diagnostics, Branchburg, NJ) and according to manufacturer's instructions.

Statistics. Determination of between groups statistically significant differences was carried out by Man-Whitney U test, Kruskal -Wallis ANOVA and Chi Sq test, comparison of paired data at different time points - by Wilcoxon's signed rank test, and evaluation of significant correlations - by Spearman rank order correlation test (Statistica software, v.5.5).

Immunological and virological criteria for the effect of HAART and patients group definition used in the study. The following immunological criteria were used to characterize the effect of HAART during the examined period:

1. Baseline CD4 AC, taken at the start of HAART
2. CD4 AC attained three months after the beginning of HAART;
3. Maximum CD4 AC: the highest CD4 AC attained in the course of 24 months of HAART;
4. Time to reach the maximum CD4 AC after the beginning of HAART;
5. CD4 AC reached at the endpoint (24th month of HAART)
6. Absolute increase of CD4 AC: the difference between the endpoint and baseline CD4 AC;
7. Maximal absolute increase of CD4 AC: the maximal difference between CD4 AC in the course of therapy and the baseline CD4 AC;
8. Mean velocity of CD4 AC increase: the difference between the endpoint and baseline CD4 AC divided by the number of months on HAART (24).

A maximum increase of CD4 AC with more than 100 cells/μl in comparison to baseline level was defined as a positive response to HAART. Maintenance of this response at 24th month was considered as response sustainability. Changes in CD4 AC at 24th month <100 cells/μl as compared to baseline was defined as a bad response to the HAART (treatment failure).

The effect of HAART on the viral burden was characterized by the following virological criteria:

1. Presence of anti-viral effect: decrease of VL with >1 log RNA copies/ml;
2. Absence of anti-viral effect: impossibility to reduce VL under 500 RNA copies /ml, or impossibility to reduce VL with at least 1 log two months after the beginning of HAART;
3. Complete virological control: reduction of VL < 2.7 log RNA copies/ml at least at one examination.
4. Maintenance of virological control: VL < 2.7 log RNA copies /ml at the endpoint.
5. Failure of virological control: VL increase > 2.7 log RNA copies/ml in two consecutive examinations.

RESULTS

1. Defining subgroups of HIV+ patients according to baseline CD4 AC.

Three subgroups were defined in the studied cohort of patients, based on the heterogeneity of the baseline CD4 AC. The average baseline CD4 count was 115 cells/μl but it ranged from 2 to 431 cells/μl. According to the cut-off values obtained after the categorization of baseline CD4 AC, the patients fell into the following three groups (Fig. 1):

- L - patients with low baseline CD4 AC (< 30 cells/μl)
- M - patients with moderate baseline CD4 AC (30-150 cells/ μl)
- H - patients with relatively high baseline CD4 AC (> 150 cells/μl)

The principal baseline immunologic (percentage and count of CD4 T, count CD38 molecules expressed of CD8 T lymphocytes) and virological parameters (viral load) of the subgroups are presented in Table 1.

No significant differences were established between the defined subgroups according to age, presumable disease duration or baseline viral load (KW p > 0.05 for all). Immune activation assessed according to the quantitative expression of CD38 on activated CD8 T cells (CD8+CD38+) was significantly different only between groups L and H (p < 0.05, MW).

2. Assessment of the immunological response to HAART in the defined subgroups of HIV+ patients.

After 2 months of HAART (a period, characterized with redistribution of CD4 T cells), the highest average CD4 AC was attained in the group with highest baseline CD4 AC (H): 391 cells/μl, followed by group M: 200 cells/μl and group L:78 cells/μl, with statistically significant differences between groups (p<0.0001, KW Annova).

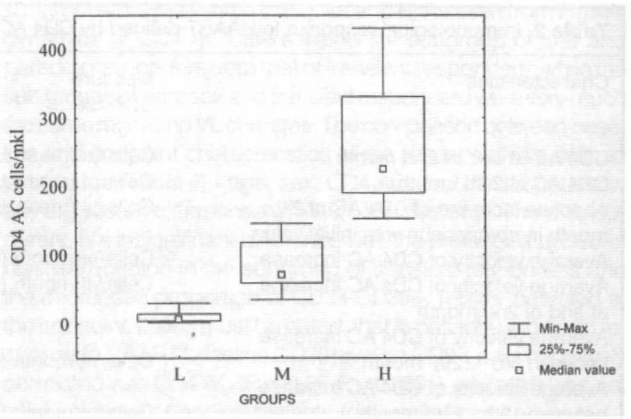

Fig. 1. Distribution of CD4 AC in the patients' subgroups defined according to baseline CD4 AC (L, M, H). Box and whiskers denote the median; 25th-75th percentile and min-max values.

The maximum CD4 AC was reached on the 24th month of therapy for the three subgroups L, M, and H: 222, 318, and 472 cells/μl, respectively. The comparison between the CD4 AC values attained in each subgroup on 24th month, showed statistically significant differences between L and H (p<0.001, MW) and between M and H (p<0.01, MW), but not between L and M (p>0.05, MW). The analysis of the individual changes of CD4 AC showed that the maximum CD4 AC values were attained relatively earlier in subgroup H (up to 12th month in over 35 % of the patients), followed by group M (up to 18th month in over 25% of the patients), (Table 2).

Noteworthy, the mean velocity of CD4 AC increase in subgroups L, M and H was very close (23, 26 and 40 cells/μl/month, respectively). The dynamics of CD4 AC was characterized by an abrupt increase between 3rd and 6th month, followed by a considerably delayed increase at 6th month (subgroups L and M) and at 9th month (subgroup H). Thereafter a tendency of slow and constant increase was maintained in all subgroups (Fig.2A). The comparison of the velocity of CD4 AC increase at each examined time point did not show statistically significant differences between the subgroups (p>0.05, KW Annova). Moreover, the velocity of CD4 AC increase compared separately for the periods 2 - 12 month and 12-24 did not show statistically significant differences between the subgroups, either (p>0.05, KW Annova). The only difference was the more extended period of high velocity CD4 AC increase in subgroup H.

To exclude a possible effect of the baseline CD4 AC, an additional parameter was examined: the absolute increase of CD4 AC. The curves obtained for the three subgroups were similar, without significant differences at the examined time points (p>0.05, KW Annova). The average absolute CD4 AC increases were 151, 182 and 184 cells/μl in the groups L, M and H respectively. (Fig. 2 B)

Table 1. Baseline characteristics of the subgroups HIV+ patients (L, M and H).

Characteristics	Measure	Group L CD4<30	Group M 30<CD4<150	Group H CD4>150
N of patients	number	21	17	17
Sex (male: female)	%	81:19	59:41	53:47
Presumable infection duration	Months, mean (median)	60 (36)	65 (42)	66 (60)
Age	Years, mean (median)	37 (38)	37 (36)	37 (37)
Baseline CD4 AC	Cells/μl (mean ± SD)	11 ± 10	81 ± 42	260 ± 92
Quantitative CD38 expression on CD8 T cells	CD38 ABC (mean ± SD)	12885 ± 6386	11822 ± 8943	9646 ± 7255
Baseline VL	Log RNA copies/ml (mean ± SD)	5.4 ± 0.54	5.2 ± 0.56	4.9 ± 1.2

Table 2. Immunological response to HAART defined by CD4 AC changes, in the subgroups HIV+ patients (L, M and H)

Characteristics	Measure	Group L CD4<30	Group M 30<CD4<150	Group H CD4>150
CD4 AC at end of 2nd month	Cells/μl (mean ± SD)	78 ± 52	200 ± 84	391 ± 175
CD4 AC at 24th month	Cells/μl (mean ± SD)	222 ± 145	318 ± 138	472 ± 204
Absolute increase of CD4 AC at 24th month in comparison with initial value	Cells/μl (mean ± SD)	151 ± 52	182 ± 41	184 ± 41
Average velocity of CD4 AC increase	Cells/μl/month (mean ± SD)	23 ± 20	26 ± 17	40 ± 26
Average velocity of CD4 AC increase at end of 2nd month	Cells/μl/month (mean ± SD)	67 ± 40	119 ± 80	132 ± 90
Average velocity of CD4 AC increase between 3rd - 12th month	Cells/μl/month (mean ± SD)	24 ± 13	16 ± 3	17 ± 6
Average velocity of CD4 AC increase between 12th - 24th month	Cells/μl/month (mean ± SD)	17 ± 13	12 ± 7	16 ± 10

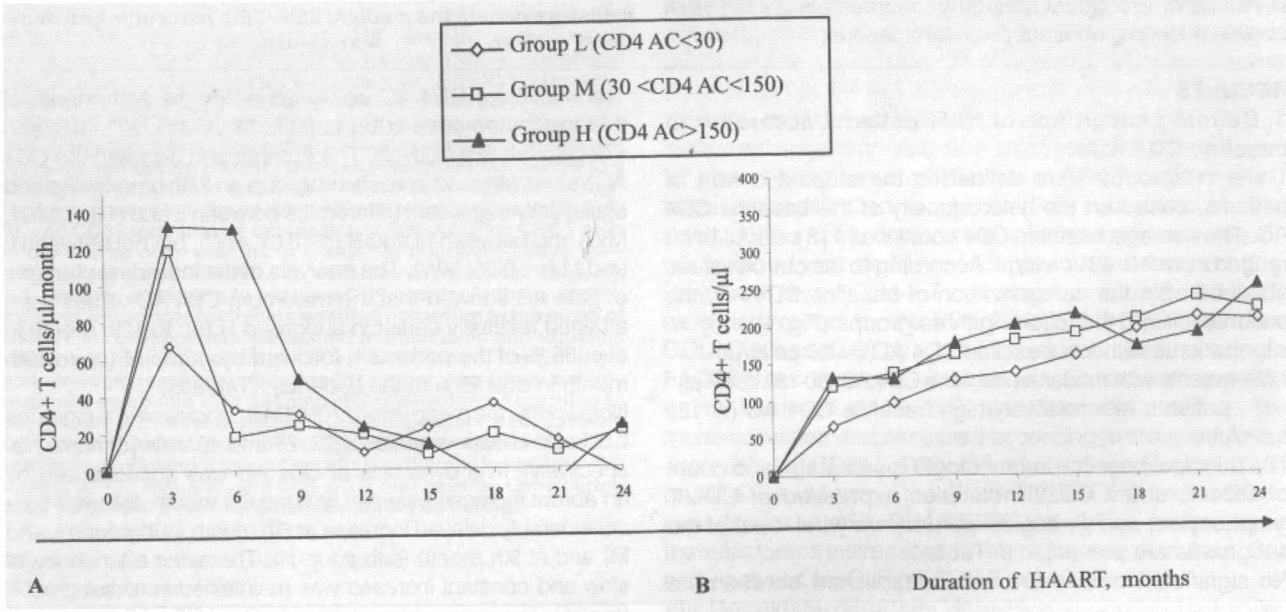

Fig. 2. Average velocity of CD4 AC increase (A) and average absolute CD4 AC increase (B) at the studied time points in patients' subgroups L, M and H.

According to the accepted definition, patients with good immunological response to HAART in subgroups L, M and H comprised 70%, 65% and 88% respectively. This effect was sustained in 100%, 87% and 86% patients from the relevant groups. Thus, results for the immunological effect of HAART as defined by CD4 AC changes were not statistically significant between the subgroups (Chi Sq >0.05).

3. Assessment of the antiretroviral effect of HAART in the subgroups of HIV+ patients.

Antiretroviral effect of HAART was evaluated on the basis of the observed changes in VL and immune activation (assessed by the quantitative expression of CD38 on CD8 T lymphocytes). The individual values of VL determined at baseline, on 12th and 24th - month of HAART were not significantly different between subgroups L, M, H (p>0.05, KW, data not shown). The average VL and immune activation values in the subgroups were also similar in the end of the examined period (Table 3).

After 2 months of HAART, about 95% from L patients, 100% from M patients and 94% from H patients had a positive antiretroviral effect. Percentages of patients with full antiretroviral effect induced by HAART at specified time points are presented in Fig. 3 . The highest percentage of patients with good antiretroviral response induced by HAART was observed after 6 months of HAART: 65%, 70%, 76% for groups H, M, and L, respectively. At the endpoint, however, the antiretroviral effect was lost in a proportion of the patients. The antiretroviral effect was preserved in 92%, 83% and 80% of the patients in sub group H, M, L respectively. The comparison of the antiretroviral effect of HAART for the individual patients at all specified time points did not show statistically significant differences between the subgroups (p>0.05, KW Annova).

Table 3. Average VL and immune activation values in the subgroups HIV+ patients (L, M and H), at endpoint

Characteristics	Measure	Group L CD4<30	Group M 30<CD4<150	Group H CD4>150
Average VL after 24 months HAART	Log RNA copies/ml (mean ± SD)	3.3 ± 1.04	4.02 ± 0.54	4.3 ± 0.67
Average CD38 antibody-binding capacity at endpoint	CD38 ABC (mean ± SD)	2288 ± 1593	1927 ± 882	1775 ± 1108

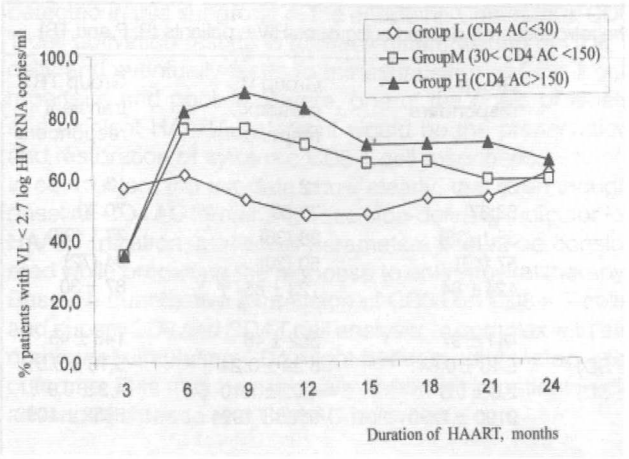

Fig. 3. Percentage of patients with complete antiretroviral effect of HAART (VL < 2.7 log HIV RNA copies/ml) in the subgroups defined according to baseline CD4 AC (L, M, H).

4. Defining of patients' subgroups on the basis of the complex immunological and antiretroviral effect of HAART.

The highest coincidence between an immunological and antiretroviral effect of the therapy was observed in subgroup H (70% of the patients), followed by group L (45%), and group M (30%). However, a statistically significant correlation between the immunological and antiretroviral effect of therapy was not established (Chi Sq >0.05). In all three subgroups, patients with restoration of CD4 AC without concomitant lasting reduction of VL were observed (25%, 35% and 24 % in L, M and H subgroups respectively). The opposite effect, inhibition of viral replication without CD4 AC increase was observed in only one patient from subgroup H.

Based on the complex immunological and antiretroviral effect of HAART, patients were grouped retrospectively as follows:

- responders, R: Patients with sustained complete (immunological and virological) response to HAART: absolute CD4 AC increase > 100 cells/μl and VL< 500 copies/ml on 24th month;
- paradox responders, P: Patients with sustained immunological effect of HAART (absolute CD4 AC increase > 100 cells/μl on 24th month), but failure of VL control at 24 months;
- transient responders, TR: Patients with transient or without immunological and virological effects of HAART.

Dynamics of CD4 AC increase and proportions of patients with anti-viral response in the each of retrospectively defined subgroups are presented in Fig. 4A and B. As seen from Fig.4A, dynamics of CD4 AC differentiated the subgroup of true and paradox responders from that of transient responders, while the sub groups of paradox and transient responders were very much the same regarding VL changes. The comparison between baseline and endpoint characteristics of the retrospectively defined subgroups (Table 4) - age, sex, CD4 AC, and VL did not show any significant differences (p>0.05, KW Annova). The only apparent, but insignificant difference was the prolonged period of disease duration in the subgroup of transient responders and the decreased proportion of CD8+CD38+ T cells detected at the endpoint. It also must be noted, that in patients with good response to HAART baseline CD38 level on CD8 T cells negatively correlated with CD4 AC (R=-0.65, P <0.0001), while this was not valid for non-responding patients (data not shown).

DISCUSSION

HIV infection results almost inevitably in progressive destruction of the immune system and impairment of immune function (11;18;27). At present, HAART is the only working therapeutic strategy against HIV. In recent years, investigations on HIV-infection and HIV-specific immune response have lead to a better understanding of HIV-induced immunodeficiency and the possibilities for its restoration by means of HAART (14;23;30;33). The ability to predict the effect of HAART is a key point in defining the appropriate moment for HAART initiation as well as for changes in the treatment regimen (29). The most popular criterion used to determine HAART starting point is CD4 AC, since it reflects the degree of immune system damage. This concept reflects the correlation between the number of CD4 T cells and the appearance of opportunistic infections, which spectrum and incidence increase with the development of immune deficiency. Opportunistic infections are usually manifested when CD4 count drops below 500 cells / μl. Thus, the clear-cur association between CD4 count and the incidence of opportunistic infections and malignant diseases is the rationale for using CD4 count as criterion for starting HAART. Two views for starting HAART exist:

Starting therapy as early as possible while the immune system is still preserved to achieve a quick and more complete immune recovery or delay of therapy to avoid toxicity and side effects of HAART as well as the early appearance of resistant strains. According to the European and USA guidelines, in asymptomatic patients HAART is started at CD4 count between 500 and 350 cells/μl depending on viral load (1;28). At the other hand, the value of 200 CD4 cells/μl has been accepted as a critical line for treatment initiation according to WHO (13).

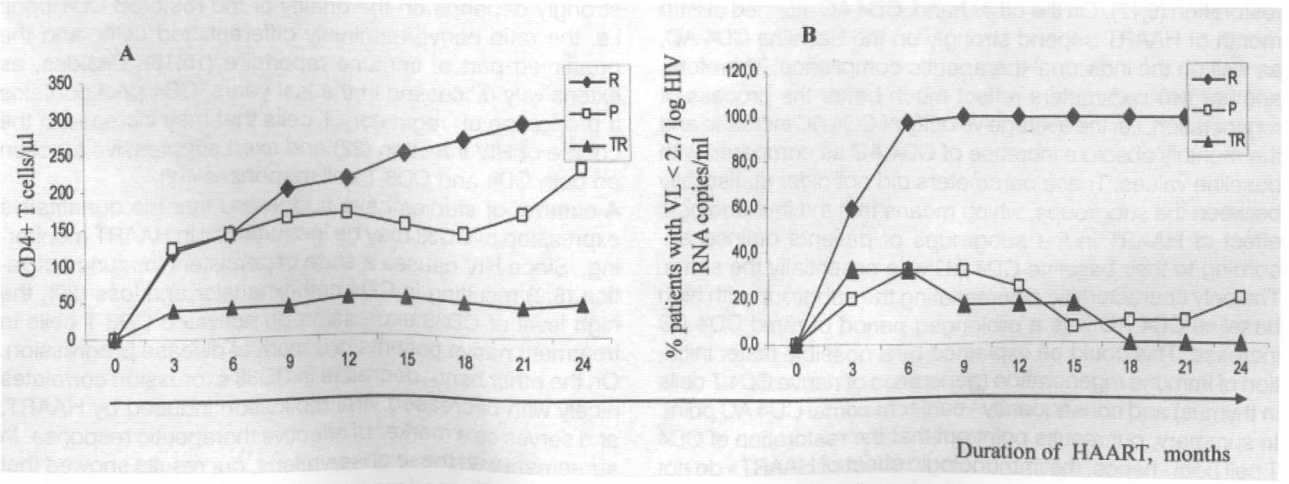

Fig. 4. Immunological and antiretroviral effect of HAART in the retrospectively defined groups (R, PR and TR): (A) absolute CD4 AC increase at the studied time points; (B) Percentage of patients with with complete antiretroviral effect of HAART (VL < 2.7 log HIV RNA copies/ml).

Table 4. Comparison between baseline and endpoint characteristics of the retrospectively defined subgroups HIV+ patients (R, P and TR)

Characteristics	Measure	Group R responders	Group P paradox responders	Group TR transient responders
N of patients	number	30	12	13
Sex (male:female)	%	63:37	70:30	70:30
Average age	Years, mean (median)	35.1 (36)	38 (36)	37.1 (37)
Presumable infection duration	Months, mean (median)	57 (49)	50 (30)	94 (72)
Average CD4 AC at baseline	Cells/μl (mean ± SD)	124 ± 84	123 ± 85	87 ± 30
Average CD4 AC at endpoint	Cells/μl (mean ± SD)	441 ± 97	352 ± 48	146 ± 45
Average VL before HAART	Log RNA copies/ml (mean ± SD)	5.13 ± 0.64	5.29 ± 0.24	5.16 ± 0.51
Average VL after 24 months HAART	Log RNA copies/ml (mean ± SD)	2.3 ± 0.3	4.02 ± 1.10	4.3 ± 0.9
Average CD38 antibody-binding capacity at baseline	CD38 ABC (mean ± SD)	9190 ± 1290	9506 ± 1994	5652 ± 1092
Average CD38 antibody-binding capacity at endpoint	CD38 ABC (mean ± SD)	1800 ± 800	2730 ± 550	3419 ± 400

In the recent study, the cut-off CD4AC values (35 and 150 cells/μl) used to define patients subgroups reflected the generally low baseline values of the examined HIV+ cohort (only 22% of patients had CD4 AC >200 cells/μl and only 10% - CD4 AC >350cells/μl). Therefore, we asked whether a further differentiation of lower CD4 AC was predictive about long term HAART effects.

As expected, HAART administration lead to CD4 AC increase as early as the end of 2nd month, as a result of reduced viral replication, decreased immune activation and release of trapped CD4 cells from the lymphatic tissues (3-5;10;23) Although this effect was observed in all subgroups, the differences between CD4 AC remained statistically significant. These results were not unexpected, having in mind the similar baseline viral burdens in the three subgroups, and suggest that the absolute count of circulating CD4 cells is proportional to the total CD4 T cell pool. Further on, in patients with advanced HIV disease and extreme immune activation, the initial CD4 AC increase may only reflect a more intensive CD4 cell trapping in lymphoid tissues. Therefore, CD4 AC values attained after 24 months of HAART should reflect much more accurately the immune system restoration potential. According to our results, patients from the subgroup with highest baseline CD4 AC (H) attained a statistically higher CD4 AC in 24 months of HAART, while the subgroups M and L (baseline counts < 150 cells/μl) did not differ statistically. These results are in line with literature data showing that the peripheral blood concentration of 150-200 CD4 cells/μl is critical for immune restoration (6;17). On the other hand, CD4 AC attained at 24th month of HAART depend strongly on the baseline CD4 AC, as well on the individual therapeutic compliance. Therefore, another two parameters reflect much better the process of regeneration, i.e. the average velocity of CD4 AC increase and the monthly absolute increase of CD4 AC as compared with baseline values. These parameters did not differ statistically between the subgroups, which means that the immunologic effect of HAART in the subgroups of patients defined according to their baseline CD4 AC was essentially the same. The only characteristic differentiating the subgroup with high baseline CD4 AC was a prolonged period of rapid CD4 AC increase. This could be explained by a possible faster initiation of immune regeneration (generation of naïve CD4 T cells in thymus) and consequently - earlier maximal CD4 AC point. In summary, our results point out that the restoration of CD4 T cell pool - hence, the immunologic effect of HAART - do not depend strongly and solely on the baseline CD4 AC.

Analysis of the antiviral effect of HAART in the three subgroups strengthened the above observations. At 24th months of HAART, VL was not statistically different between the subgroups defined according to baseline CD4 AC. Moreover, the proportion of patients with complete and lasting antiretroviral effect of HAART was similar in the three subgroups. In other words, the complex immunologic and virologic effect of HAART did not seem to depend significantly on baseline CD4 AC, at least in the range 0-400 cells/μl. At the same time, it should be kept in mind that only in subgroup H the high proportion of cases with immunologic effect of HAART had a combines virologic effect of therapy (%). For the rest of the patients (with CD4 AC< 150cells/μl), in a great proportion of the cases a paradoxical effect was observed, ie increase of CD4 AC accompanied with persisting high level viral replication. A possible explanation for continued immunological success accompanied by viral replication may be a continuous partial suppression of HIV replication, or primary impaired fitness of drug resistant viruses (26). Recent data suggest that patients with „discordant" response have a quiescent immune with prevalence of resting CD4 cells (21). However, the issue about the real immune restoration of these patients remains open. In any case it is believed that a long term immunological success could not be sustained without a concomitant virological response.

The retrospective grouping of patients (R,NR,P) based on the complex (immunological and antiretroviral) effect of HAART gave us a better possibility to assess the dynamics of immune restoration. Indeed, racing CD4 T cells counts after therapy do not necessarily predict immune competence. It has been shown that a better immunological outcome strongly depends on the quality of the restored CD4 pool: i.e. the ratio naïve/terminally differentiated cells, and the preserved part of immune repertoire (15;19). Besides, as extensively discussed in the last years, CD4 pool contains a proportion of regulatory T cells that may increase in the course of HIV infection (32) and exert suppressive function on both CD4 and CD8 T cell responses (12).

A number of studies have suggested that the quantitative expression of CD38 may be instrumental in HAART monitoring. Since HIV causes a state of persistent immune activation (8;9) resulting in CD4 cell exhauster and loss (20), the high level of CD38 expression on activated CD8 T cells in treatment naïve patients is a mark of disease progression. On the other hand, decrease in CD38 expression correlates nicely with decreased viral replication induced by HAART, and serves as a marker of effective therapeutic response. In agreement with these observations, our results showed that in patients with good response to HAART, baseline CD38 levels negatively correlated with CD4AC ($R=-0.65$, $P<0.0001$), while this was not valid for non-responding patients. Furthermore, a decreased proportion of CD8+CD38+ T cells was

detected in this subgroup at the endpoint. Continuous CD8 T cell activation results in terminal differentiation of CD8 T cells and eventually leads to the exhaustion of CD8 T cell repertoire and pool. Therefore, one of the goals of timely and efficient HAART treatment would be the preservation and restoration of cytotoxic CD8 T cell responses.

In conclusion, the our data show clearly, that even though baseline CD4 AC remains the decision-defining indicator for HAART initiation, additional parameters should be considered while predicting the response to antiretroviral therapy. Baseline quantitative expression of CD38 on CD8+ T cells and subset CD8 and CD4 T cell analysis, in complex with the number of circulating CD4 might better predict therapeutic outcome. This may be especially important in patients with advanced disease and CD4 AC below 400.

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BK-POLYOMAVIRUS (BKV) EXCRETION AND VIREMIA IN WOMEN WITH PRIMARY AND RECURRENT GYNECOLOGICAL MALIGNANCIES

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SUMMARY

Reactivation of latent viral infections is one of the major causes for morbidity and mortality among patients with cancer. Human polyomavirus BK (BKV) causes latent infection and is frequently reactivated in immunocompromised hosts. Apart from patients with leukemic malignancies, the disease pattern among individuals with other types of oncologic diseases is not investigated. We examined 109 women with tumors of the genitalia separated into two groups: with primary neoplasia and a group receiving diverse kinds of therapeutic procedures. Both groups had high and similar values of BKV urine reactivation, over 60%, and a small percentage (>10%) of viremia. We imply that the general immune dysfunction caused by the tumor itself, rather than therapeutic approach is a major factor for BK viral reactivation in this group of patients.

Key words: human polyomavirus, BK virus, BKV, LUX real-time PCR

INTRODUCTION

The human polyomaviris BK (BKV) is a latent virus, the reactivation of which is an example of immune impairment. Its involvement into the development of severe clinical entities in the field of renal transplantation due to immunosuppressive regimens is well established. It is the agent that causes Polyomavirus Associated Nephropathy (PVAN) leading dysfunction and loss of the transplanted kidney (Hirsch, 2003). Immune dysfunction but of a different type is observed in patients with oncologic diseases. These pathologies are connected with deficiency due to disturbance in antibody or cell-mediated response or the non-specific factors of the immunity. On the other hand their cytotoxic therapy can also lead to impairment. This is an artificially induced deficiency that requires special modulation of the immune system. Specific feature in patients with diverse malignancies is the reactivation of latent bacterial, fungal, viral and parasitic infections which additionally require immune-stimulation and etiologic therapy (Bojkov B., 2000).

Morbidity and mortality in patients with malignancies is increased by viral infections. These are mostly reactivations of asymptomatic latent viruses. The main risk factor for clinically relevant disease is the profound disruption of cellular immune response. Duration and severity of the chemotherapy is of a lesser importance. The risk of viral complications rises significantly in the presence of sustained suppression of T-cell function (Sandherr, 2006).

BKV reactivation and disease etiologies are best studied in patients with hematological malignancies. In such patients BKV may cause diverse clinical conditions, requiring specific therapy and monitoring. The outcome of this reactivation varies widely and is dependent on the intensity and duration of the T-cell induced disturbance. Most frequent is the hemorrhagic cystitis but tissue invasion and pathogenesis of the infection in such cases remains unclear (Wade, 2006). Another symptom is hematuria, described in children with different leukocyte malignancies and it is a reason for the long-lasting care and suspending the chemotherapy (Cheerva, 2007).

Except hemorrhagic cystitis and hematuria BKV may cause also tubulo-interstitial nephritis in patients with acute lymphoblastic anemia (Inaba, 2007). BKV reactivation and development of tubulo-interstitial nephritis is described in children with hairy cartilage hypoplasia and Hodgkin lymphoma and in patients with leukemic infiltration of the kidney. Nevertheless this disease due to BKV reactivation in the natural kidney is a rare event (Stracke, 2003). In older patients receiving chemotherapy for chronic lymphocytic leukemia BKV reactivation may cause pneumonia (Devine S., Wingard J., 1994).

BKV is also supposed to be involved into the development of some human urothelial tumors but its role is not well established. BKV DNA is not integrated and there is no difference in the viral copy number in normal and oncologic tissue. That is why no direct causative role of BKV for the malignancies of the small pelvis and kidneys can be proposed (Knoll, 2003).

Our objective was to examine the role of BKV reactivation and pathogenic potential in patients with different than the leukemic oncologic etiologies. Moreover we aimed at therapeutic procedures as they could be factors for increased viral shedding and disease development.

MATERIALS AND METHODS

Patient groups, urine and blood collection

The investigation included 109 female patients with malignancies of the reproductive system. For a period of nine months (IV 2007 to X 2007) a total number of 218 paired samples (109 urines and 109 sera) were collected. They were taken randomly during the stay of the patients at the Specialized Hospital for Active Treatment of Oncologic Diseases, EAD. Forty-seven of them were subjected to therapeutic procedure of a different kind. The rest 62 were with primarily diagnosed tumors and did not receive treatment. The last group was also used as a negative control. The clinical records were examined for any existing urinary complications or systemic diseases.

Twenty to 50 ml of fresh voided first or second morning urine was picked into sterile containers (Corning, Corning Inc., Cat No 43082, USA) supplied with absolute alcohol (Merck, KGaA), slowing down the development of bacterial flora (Dhundee and Rigby, 1990). Blood was placed into vacutainers (Becton-Dickinson, BD Vacutainer K2E, 3 ml, USA), containing EDTA as an anticoagulant.

Types of gynecological malignancies and therapeutic procedures

Four common types of malignancies, distributed among the patients were classified: Carcinoma of the vulva, endometria, uterine cervix and ovary. The pathological characteristics of the tumors varied as well as the grade and cellular alterations but where possible were taken into the interpretation of results. The type of therapy also varied and consisted of the following main strategies: gamma therapy, operation of the tumor, chemotherapy (variable dosage of the cytostatics) and LAP.

Preliminary preparation of urine and blood

For obtaining urine sediment cells for BKV molecular detection, the semi-automated Bales method was applied (Koss LG, 1996). In brief it had the following steps: urine was centrifuged at 1500 rpm, the supernatant was discarded and to the separated sediment a mixture from 3 to 5 ml of 70% ethanol with 2% of Polyethylene Glycol was added. After another centrifugation at the same speed and again removing the supernatant, the deposited urothelial cells were washed with PBS saline buffer and used for DNA extraction.

Freshly collected peripheral blood was initially left at 4°C for approximately half an hour, until the natural separation of the cellular components (deposited at the bottom of the vacutainer) from the plasma. It was carefully removed and centrifuged at 13 400 rpm for additional separation of the cellular debris and used immediately for molecular purposes or stored at -70°C until analysis.

ABBREVIATIONS USED IN THIS PAPER:

BKV - human polyomavirus BK, PCR - polymerase chain reaction, LUX real-time PCR - Light Upon Extension real-time PCR, PVAN - Polyomavirus Associated Nephropathy

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DNA extraction and PCR

Extraction of DNA was performed by in-house modifications of the 25: 24: 1 phenol/chloroform/iso-amyl alcohol method (Invitrogen, cat No 155593-049, USA) in respect of the tested material (urine, plasma). All the extraction procedures were held along with the proper extraction controls.

PCR for BKV detection was performed using specific primers for VP1 genomic region (327-1/327-2) (Jin, 1993) and in-hose developed and optimized pair named Bk-1/Bk-2 in a qualitative reaction. In-house primer sets were derived from the nucleotide sequence of the N-terminus of the BK viral T antigen (BK stain Dun, accession number V01108) (Kalvatchev, 2007). For insurance of their specificity, all of the reactions for primer optimization and PCR were run with the appropriate positive BKV DNA controls. BKV primers are described in Table 1.

Table 1. Features of the used primer sets

Name	Position	Annealing t°*	Sequence (5'-3')	Amplicon**
BK virus Dunlop strain (accession number in Genebank V01108)				
327-1	1630-1649	55°C	CAA gTg CCA AAA CTA CTA AT	327 bp
327-2	1956-1937	55°C	TgC ATg AAg gTT AAg CAT gC	
Bk-1	2992-3011	60°C	ATC CAg CCT TTC CTTCCA TT	218 bp
Bk-2	3090-3211	60°C	CTg TTC CTA AAA ACC TgC CAA	

* In-house optimized annealing temperatures
** Size of the amplified DNA product

The amplification reactions were held in 50 μl volume with 10X reaction buffer, 1.5 mM magnesium chloride (ABgene), 200 μM from each of the four nucleotides (USB) and 1.25 U thermo stable Taq polymerase (5U/μl, Cat. No. 10342-020, Invitrogen, USA). A total of 5 μl (~0.5 μg) extracted DNA and 600 nM of each primer were also added to the mixture. The amplification ramp included initial step of denaturation at 94°C for 5 min followed by three-step cycle consisting of denaturation at 94°C for 30s and extension at 72°C for 1 min. The annealing was for 30s and its temperature varied in respect to the used primers (Table 1). Aliquots (25 μl) of each PCR product were run on 2% agarose gel stained with ethidium bromide for 80 min at 100 V (room temperature) along with 100 bp DNA MW ladder (Cat. No. 15628-019, Invitrogen, USA). All the PCR assays included positive and negative controls and were performed in DNA Engine Opticon 2 (MJ Research, USA).

Statistical analysis

Statistical analysis was carried out using the Pearson criteria, with a value of 0.05 considered as a threshold of significance. Data between the study groups and different variables was compared with one-way analysis of variance- ANOVA. The statistical calculations and the estimation of frequencies were carried out using the statistical software package SPSS 9.0 (SPSS Inc.).

RESULTS AND DISCUSSION

PCR results

The positive urine and sera samples revealed abundant amplicons of 327 bp for the primer pair 327-1/327-2 and 218 bp for the set Bk-1/Bk-2. They were always compared to the amplification of the BK DNA controls and no positive results were observed in the negative controls. The sensitivity and specificity of both primer sets was estimated to be equal (Data not shown). The results from the gel-electrophoresis of the amplified products are shown at Fig. 1.

Results obtained from the patients with primary gynecological malignancies

The first group of patients included 62 women with primary malignancies and mean age of approximately 53 years. Clinical records showed four common types of diseases: carcinoma of the endometria (n=24/38.7%), carcinoma of the uterine cervix (n=23/37.1%), carcinoma of the ovary (n=10/16.1 %) and carcinoma of the vulva (n=5/ 8.1%). They did not receive therapy for and the cellular types varied greatly, that is why it was very difficult to be classified on a histological base. No urologic complications or diseases were observed according the clinical records.

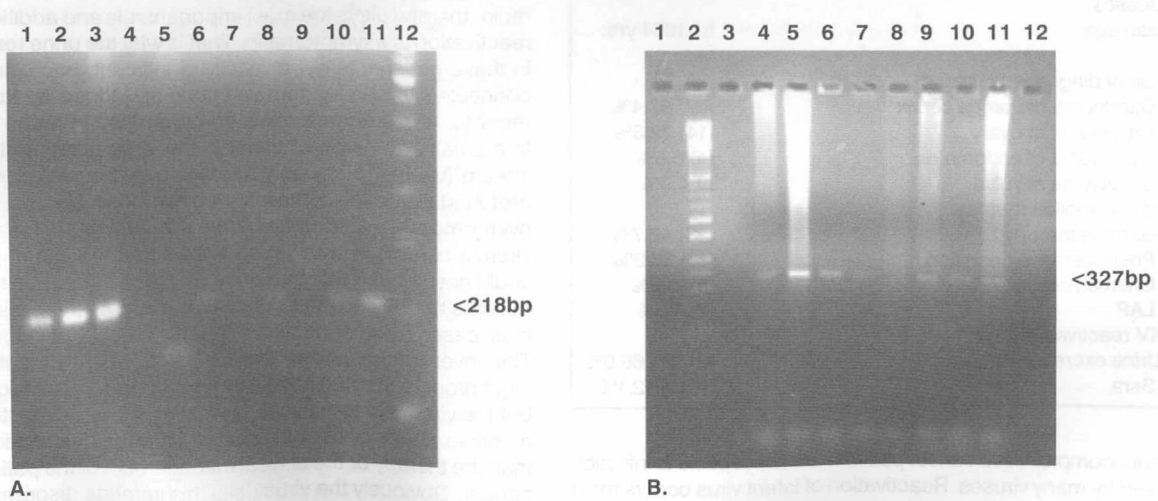

Fig. 1. Gel-electrophoresis of the amplified BKV PCR products. A. Amplified products with the primers Bk-1/Bk-2 (size 218 bp) from urine and sera of patients with gynecological malignancies. 1- positive BKV control; 2, 3-positive urine samples; 4, 5-negative urine samples; 6, 8, 10, 11-positive sera; 7-negative sera; 9-negative amplification control; 12-MW marker (100 bp); B. Amplification products obtained by 327-1/327-2 primers (size 327 bp): 1, 3-negative controls; 4-positve control; 5, 6, 7-positive urine samples; 8-negative urine; 9, 10, 11-positive sera; 12-empty; 2- MW marker (10 kb).

The PCR on urine showed that 60.7% (n=38/62) of them were shedding BKV at the time of collection of the samples. The paired sera showed a percentage of 24.1% (n=15) for viremia, as all the viremic patients had at the same time viruria. No one of the patients with virema was absent of BKV in urine. Intensity of viruria influenced the viremia (p=0.03). The main characteristics of this group and the results could be summarized in Table 2.

Table 2. General features of the patients with primary tumors of the genitalia

Group of 62 patients with primary gynecological malignancies	
Mean age	53.4912 yrs.
Type of diagnosed disease	
1. Carcinoma of endometria	(24) 38.7%
2. Carcinoma of uterine cervix	(23) 37.1%
3. Carcinoma of ovary	(10) 16.1%
4. Carcinoma of vulva	(5) 8.1%
BKV reactivation	
Urine	(38/62) 60.7%
Sera	(15/62) 24.1%

Results from the group of the patients with malignancies subjected to therapy

This group included 47 women with mean age approximately 51years. They had the following distribution of different tumors: carcinoma of uterine cervix (n=27/57.4%), carcinoma of ovary (n=14/29.8%), carcinoma of endometria (n=4/8.5%) and carcinoma of vulva (n=2/4.3%). These patients were subjected to various types of therapy which were grouped into the following strategies: gamma therapy (n=23/48.9%), operative removal (n=17/36.2%), chemotherapy (n=5/10.6%) and LAP (n=2/4.3%). The most prevalent were gamma therapy followed by the operative procedures. At the same time the percentage of virus shedding was 66% (n=31/67) and the detected BK sequences in blood was 12.1% (n=6/47). Again, all of the patients with viremia, had viruria, and no one with viremia was without viruria. The correlation between the viremia and viruria was as the previous group and no significant influence of the therapy on the BKV reactivation was observed. The characteristics of these patients are summarized in Table 3.

Table 3. General features of the patients undergoing anti-tumor therapeutic procedure

Group of 47 patients undergoing therapy for the oncologic diseases	
Mean age	51.1064 yrs.
Type of diagnosed disease	
1. Carcinoma of uterine cervix	(27) 57.4%
2. Carcinoma of ovary	(14) 29.8%
3. Carcinoma of endometria	(4) 8.5%
4. Carcinoma of vulva	(2)4.3%
Type of applied therapy	
1. Gamma therapy	(23) 48.7%
2. Post-operative condition	(17) 36.3%
3. Chemotherapy	(5)10.6%
4. LAP	(2) 4.3%
BKV reactivation	
1.Urine excretion	(31/47) 66.0%
2. Sera	(6/47) 12.1%

Immunocompromised cancer patients are susceptible to infection caused by many viruses. Reactivation of latent virus occurs most often as a sequel of cytotoxic therapy, when the cell-mediated immunity especially cytotoxic responses, the major host protective defense are diminshed. The resolution of these infections is dependent on the control of the malignancy and the ability of the patient to mount and adequate immune response. The most serious morbidity results from active infection by members of the herpes simplex family, which are problematic in patients with all types of cancer. But in the field of herpes viruses there are several highly active agents against them and this has resulted in reduced morbidity and mortality (Devine, 1994).

The situation is quite different with the human polyomavirus BK. There are not anti-viral drugs, and the mechanisms which allow virus to reactivation and disease are not clear. Most of the surveys are conducted on patients with leukemia undergoing bone marrow transplantation. Symptomatic disease was connected with hemorrhagic cystitis, hematuria or tubulo-interstitial nephritis (Cheerva, 2007; Inaba, 2007; Stacke, 2003). In most cases treatment was difficult and relies on bladder irrigation, long-lasting care and reducing the immune suppressant for immunological reconstruction (Cheerva, 2007).

That is why, we selected a group of patients with different than the leukemic malignancies oncologic diseases, subjected or not to immunosuppressive therapy. This might reveal the impact of the therapeutic and tumor induced immunosuppression on the reactivation of BKV.

As it could be seen from the results the urine excretion of BKV in both groups is high, it is similar and exceeds 60%. There are different arguments in respect to the high reactivation. Firstly, the individual factors (age) might be evaluated. Both groups have similar mean age (over 50 years) and it is proposed as a factor supporting BKV reactivation. Increase of age intensifies BKV activity (Polo, 2004). Age is similar and this could be a reason for the approximately equal level of reactivation. This individual factor alone could not be the only reason for reactivation. Another reason is possibly the immunologic surveillance of the oncologic patients. The tumor itself has the ability to suppress the immune response in the host (diminishes the functions of the T-cells, B-cells antigen presenting cells, lowers production of IL-2 as well as increases that of soluble IL-2 receptors). This is performed by not well studied mechanisms (Merck).

This is strengthened by the fact that in both groups it was proven statistically that the degree of viral shedding is not influenced by the type of the disease and the applied type therapy. This reveals that possibly the tumor breaks down the immune competence and makes the patient susceptible to reactivation of latent BKV. The immune response towards various antigens is diminished in patients with cancer. This must be distinguished from the common immune dysfunction developed by chemotherapeutics. It is antigen-specific at the initial stages of the disease but subsequently becomes generalized. Most probably this generalization causes frequent urine reactivation of latent BKV in both groups (Bojkov, 2000).

BK is a pathogen that can be reactivated during small changes of the immune competence. Thus, the immune dysfunction developed by the tumor itself rather than factors like chemo- or radio- therapy plays the most important role and additionally the reactivation is asymptomatic. That is why the urine reactivation in these patients is not clinically significant, because it is not connected to urinary complications as judged by the clinical records. It is a marker of a compromised immune function. In a small proportion of patients the virus gets into the blood stream (viremia). Probably this again is connected with more profound disruption of the immune response, because the virus overcomes the blood-urine barrier and circulate in the sera. The viremia combined with viruria shows that the immune system could not manage with that urine BK reactivation, and the viruric patients become viremic. Statistically a more intensified viruria in all cases was connected with development of viremia.

This investigation shows that BKV is frequently reactivated in large proportion of the patients with gynecological malignancies, but the virema is rare event. Moreover, the virus reactivation is a consequence of the generalized immune dysfunction rather than the therapy or the clinical management of the patients with cancer. Obviously the virus uses this immune disorder caused by the tumor to get into active replication. May be factors like radio or chemotherapy additionally may provoke the viral reactivation, but no statistical correlation was observed. Thus the reactivation of BKV in patients with gynecology malignancies is frequent but not clinically significant event and is a marker of compromised immune response.

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OCCURRENCE OF MULTIRESISTANT ENTEROBACTER CLOACAE STRAIN IN A NEUROSURGERY INTENSIVE CARE UNIT

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SUMMARY

From May to July 2007, multi-resistant E. cloacae were isolated from tracheal aspirates of three patients admitted to the neurosurgery intensive care unit at University Hospital - Pleven. These isolates were resistant to broad-spectrum cephalosporins, gentamicin and tobramycin, and susceptible to cefepime, carbapenems and quinolones. On the basis of that, an outbreak was suspected and an epidemiological study was performed. Susceptibility results showed circulation of hospital acquired strain E. cloacae resistant to extended-spectrum cephalosporins. Further molecular typing is required to determine whether a limited spread of a single strain existed and to recognize the exact types of resistance. Epidemiological data didn,t confirm outbreak and revealed colonization of respiratory tract in patients involved. Multi-resistant E. cloacae were cultured from the aspirating catheter solutions, so that the environmental sources of such isolates were determined and effective control measures were established. Despite of that, occurrence of multi-resistant E. cloacae in an intensive care unit is a great risk for emerging nosocomial infections.

Key words: multi-resistant E. cloacae, extended-spectrumcephalosporins, aspirating catheter, epidemiological study, nosocomial pathogen

INTRODUCTION

Enterobacter spp. are becoming frequent nosocomial pathogens, and β-lactam resistant strains are on the increase, mainly among isolates from intensive care units (ICUs) (12). Numerous studies have shown that the risk factors for acquiring such pathogens include the prolonged hospital stay, especially in an ICU, the serious underlying illness, immunosuppresion from any cause, extremes of age, the presence of a foreign device and prior use of antimicrobial agents in the patients involved (1, 2, 6, 9, 18, 19). Enterobacter spp. are known to cause significant nosocomial infections, including septicemia, urinary tract and respiratory tract infections (5, 7, 8, 19).

Antimicrobial susceptibility varies widely due to the diverse species within the genus (12). In general, there is the greater prevalence of resistance among isolates of Enterobacter to β-lactam antibiotics, thrimethoprim-sulfamethoxazole, and quinolones (4, 8, 9, 10, 12, 20, 21). Reports of multidrug-resistant isolates have increased during the last decade, probably as a result of the extensive use of broad-spectrum antibiotics (8). Data from the National Nosocomial Infection Surveillance System (14) reveal that more than one-third of Enterobacter spp. isolated in ICU are resistant to extended-spectrum cephalosporins.

ABBREVIATIONS USED IN THIS PAPER:

NCCLS - National Committee for Clinical Laboratory Standards, ICU - Intensive care unit, DDT - Double disk diffusion test

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In particular interest is Enterobacter cloacae resistant to extended-spectrum cephalosporins as a serious nosocomial pathogen in ICUs.

BACKGROUND

From May to July 2007, repeated isolates of E. cloacae were appeared in the neurosurgery ICU at University Hospital - Pleven. This was related to simultaneous isolation of multidrug-resistant E. cloacae strain from tracheal aspirates of three patients on the ward. Such strain had not been isolated in that ICU from October 2006 through May 2007. This finding initiated an epidemiological investigation to identify the potential reservoirs of the strain and to set up effective control measures.

MATERIALS AND METHODS

Review of case patients

The medical and microbiological records of patients whom a positive culture had been obtained were reviewed. The data collected included age, sex, underlying diseases, invasive procedures, the date and nature of specimens positive for multi-resistant E. cloacae, time between ICU admission and first positive culture, susceptibility results of the isolates, symptoms of infections, antibiotic treatment and outcome.

Bacteriological assessment

Tracheal aspirates routinely were collected from ventilated patients.

To investigate epidemiological situation different samples were obtained from environment, staff and patients.

The neurosurgery ICU in our hospital has 6 beds located in 2 rooms, each of which is connected with the nurses, station. Environmental swabs were taken from the areas around patients including sinks, beds, commodes, etc. Other environmental samples were obtained from humidifiers, tips of aspirating catheters, solutions for storing such catheters, feeding tubes and enteral nutrition solutions administered to the patients.

Specimens of personnel' hands were also cultured.

Additionally, faecal samples were collected from patients and staff members and were tested for colonization of E. cloacae.

All materials were cultured using standard microbiology techniques. Species identification was done by the Vitek2 Compact automated system (bio-Merieux).

Antibiotic susceptibility testing

Antibiotic susceptibility was determined by the disk-diffusion method according to the NCCLS recommendations (13) and by automated system Vitek2 Compact (bio-Merieux). Susceptibility to the following antibiotics were determined: ampicillin (AMP), amoxicillin-clavulanic acid (AUG), piperacillin (PIP), piperacillin-tazobactam (PTZ), cefuroxime (CXM), cefoxitin (FOX), cefotaxime (CTX), ceftriaxone (CRO), ceftazidime (CAZ), cefepime (FEB), imipenem (IMP), meropenem (MEM), gentamicin (GEN), amikacin (AMK)., tobramycin (TOB), ciprofloxacin (CIP), levofloxacin (LEV), and trimethoprim / sulfamethoxazole (TMP/SMZ). The isolates were screened for the presence of an extended spectrum β-lactamase (ESBL) by the double disk synergy method (13).

RESULTS

Identification of the index case

The initial multi-resistant E. cloacae strain was first isolated in the end of May 2006 from tracheal aspirate of 8-year child with head trauma. The strain was resistant to 3-rd generation cephalosporins, gentamicin and tobramycin. The repeated isolate was subsequently recovered from him tracheal secretion after one week.

Characteristics of the patients

A month later, multi-resistant E. cloacae isolates were simultaneous obtained from tracheal aspirates of two ventilated patients on the ward. Data about patients are represented on Table 1.

Environmental survey

E. cloacae with the same susceptibility profile as the multi-resistant strain was isolated from the aspirating catheter solution and the tip of aspirating catheter of Patient #3, and from the aspirating catheter solution of Patient #2, also. There was E. cloacae obtained from faecal specimen of Patient #1, but this isolate was susceptible to antibiotics recommended.
The other environmental tests and samples from personnel' hands were negative for E. cloacae.

Susceptibility results

MIC determination showed that all multi-resistant E. cloacae isolates had a similar resistant pattern. They were resistant (MICs in μg/ml) to PIP(>128), CXM(>64), FOX(>64), CTX(>64), CRO(>64), CAZ(>64), GEN(>16), and TOB(>16), moderately susceptible to PTZ(=64) and susceptible to FEB(=2), IMP(<1), MEM(<0,25), CIP(=1), LEV(=1), and AMK(=16). Concerning TMP/SMZ, the isolates of Patient #1 were susceptible with MIC <20 μg/ml, whereas all other isolates were resistant with MIC >320 μg/ml.
Double disk diffusion test (DDT) with ceftazidime (ceftriaxone, cefotaxime) and amoxicillin-clavulanic acid didn,t indicate the presence of an ESBL in the resistant strains.

Control measures

Recognition of the environmental sources of multi-resistant E. cloacae in the neurosurgery ICU led to special recommendation to medical and nursing staff in August 2007 aimed at preventing further cross-contamination. The mode of storing aspirating catheters in sterile solutions was ceased. Then only single-use packed catheters are administered to ventilated patients.
After this work, cultures of tracheal aspirates obtained in the patients in that ICU were negative for E. cloacae.

Table 1. Characteristics of the patients colonized with multiresistant E. cloacae

Patient #	Patient initials	Sex/Age /years/	Underlying disease	Days from admission to first isolation	Sites of isolation	Resistance pattern	Infection
1	DCG	M/8	Contusio capitis	7	LRT	A	-
2	MZI	M/15	Polyradiculoneuritis type Guillain-Barre	12	LRT	B	-
3	MLL	M/59	Contusio capitis	7	LRT	B	-

Table 2. Antibiotic resistance pattern of E. cloacae isolates

Isolates related to patient #	Date of isolation mo/day/yr	Specimens	Resistance pattern	Type
1	5/22/07	Traheal aspirate	PIP, FOX, CAZ, GEN, TOB	(A)
1	6/1/07	Traheal aspirate	PIP, FOX, CAZ, GEN, TOB	(A)
2	7/17/07	Traheal aspirate	PIP, FOX, CAZ, GEN, TOB, TMP	(B)
2	7/19/07	Aspirating catheter solution	PIP, FOX, CAZ, GEN, TOB, TMP	(B)
3	7/17/07	Traheal aspirate	PIP, FOX, CAZ, GEN, TOB, TMP	(B)
3	7/20/07	Tip of aspirating catheter	PIP, FOX, CAZ, GEN, TOB, TMP	(B)

DISCUSSION

In recent years multidrug resistant Enterobacter isolates have produced significant outbreaks in hospitals worldwide (6, 8, 9, 19). ICU patients were mainly involved in the most of the outbreaks previously described. Over a 3 months period 6 multi-resistant isolates of E.cloacae were collected from patients and environmental samples in our neurosurgery ICU. All isolates are characterized by the same antibiotic resistance pattern, except the resistance to TMP/SMZ, which is most likely plasmid mediated. We suggest that all isolates belong to one strain E. cloacae, which exhibits a cephalosporinase phenotype.
In Enterobacter spp. stable derepression of the chromosomal class C β-lactamase is the major cause of resistance to broad-spectrum cephalosporins (9, 11, 16, 17, 19). Production of extended spectrum β-lactamases (ESBLs) is an another important mechanism of resistance in enterobacters, but their prevalence is relatively lower (3,11,12,16,17). It is difficult to detect ESBLs in the presence of AmpC β-lactamase by use of DDT (2). Clavulanate may induce the AmpC β-lactamase, which may hydrolyze the indicator expanded-spectrum cephalosporins and mask any synergy arising from inhibition of the ESBL (15,23). Despite of difficulty in detecting clear resistance mechanism, on the basis of absence of any synergy effect in DDT and susceptibility to cefepime, we suggest hyperproduction of AmpC β-lactamase, caused either by induction or more likely, by selection of derepressed mutants. Further molecular typing is required to recognize the exact types of resistance.
In general, our results show circulation of hospital acquired strain E. cloacae resistant to expanded-spectrum cephalosporins. A number of general risk factors for acquisition of such strain: serious illnesses, emergency procedures, use of invasive devices, especially mechanical ventilation, prolong surgical operations and prior use of antimicrobials, particularly 3-th generation cephalosporins, were encountered in our patients.
This study, like others (5, 7, 8, 10), shows that entrobacters are able to colonize / infect hospitalized patients at a variety of sites, including the respiratory tract. Probably colonization of respiratory tract in our index patient originated from an endogenous gastrointestinal flora. The emergence of resistance obviously results from the development of resistance in susceptible organisms. Mutants with high level resistance to broad-spectrum cephalosporins are generally present at low rates in the gut of patients and selection during therapy allows subsequent overgrowth of these resistant mutants. It results in the isolation of high level resistant isolates later in the hospital stay. Many studies have been published on the emergence of organisms resistant to multiple β-lactam antibiotics during treatment with expanded-spectrum cephalosporins (8, 9, 10, 12, 21). In consistence with these, the analysis of our data suggests that the initial isolate was selected during therapy with ceftriaxone and transmitted to other ventilated patients

via the hands of medical or nursing staff. The incorrect mode of storing aspirating catheters in sterile solutions was resulted in contamination of these solutions and maintenance of permanent reservoir for further transmision.
Unfortunately, our attempts to detect gastrointestinal carriage of multi-resistant E. cloacae strain failed, but we prove that gastrointestinal tract of index patient was colonised with susceptible E. cloacae isolate. According to Kaye at all., in such patients there is approximately 19% risk for selection of resistant mutants during therapy with broad-spectrum cephalosporins.

CONCLUSIONS

In this study, an outbreak was suspected on the basis of increased incidence of E.cloacae isolates with a particular multi-drug resistance pattern. Our epidemiological results didn,t confirm an outbreak and revealed colonization of respiratory tract in patients involved. Detection of the source of this strain allowed to establish effective control measures, so that cross-contamination was prevented. Despite of that, occurrence of multi-resistant E. cloacae in our hospital and especially in intensive care setting is a great risk for emerging nosocomial infections. It is important to determine whether a limited spread of a single strain existed and also to study the mechanisms of resistance to newer β-lactam antibiotics.

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COMBINED TREATMENT OF HYDATIDOSIS WITH ALBENDAZOLE AND AN IMMUNO-STIMULATOR ISOPRINOSINE

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SUMMARY

Immuno-suppression accompanies hydatidosis in humans. The non-specific immuno-stimulator Isoprinosine and albendazole were administered to three groups of patients: post surgery, post PAIR and as conservative therapy. Evidence accumulated during the 2-5 year follow-up show that Isoprinosine is an effective supplementary treatment in patients with lung and liver hydatidosis.

Key words: Echinococcosis/Hydatidosis, treatment, albendasol, isoprinosin

INTRODUCTION

In Bulgaria, the morbidity rate of hydatid disease has shown a five-fold increase over the last two decades. It presents a public health problem. In 2004, a National Program was implemented to reduce morbidity and control the diseases among humans and animals. In 2006, 543 new cases were registered (corresponding to a morbidity rate of 6.28 ‰) /1/. The most important clinical aspects of the problem are related to early diagnosis (imaging and serological) as well as to adequate treatment (most often - surgical, which is considered radical) and follow-up. Due to the introduction of the conservative treatment with benzimidazole derivatives (mebendazole and albendazole), the therapeutic options have expanded /8/. These agents are usually administered: post surgery in order to avoid recurrent disease, combined with PAIR in cases of liver hydatidosis and as mono-therapy in inoperable cases, recurrent disease, multiple organ hydatidosis, cyst rupture and if patients refuse surgery /4/.

It is well-known, that parasitic immuno-suppression plays a role in the pathogenesis of hydatidosis. Attempts to reverse immuno-suppression by non-specific immuno-stimulation have been made in the past. Immuno-stimulants as BCG, levamisole, antistenocardin etc. have been administered combined with mebendazole /2/. In the last decade, isoprinosine has been proved an effective immuno-stimulating agent in HIV, chronic hepatitis, herpes virus and other recurrent infections as well as in hematological malignancies /3/. According to data in the literature, isoprinosine has a parasitocidal effect on hydatid protoscoleces and cysts in both in vitro experiments with hydatid protoscoleces and in in vivo experiments with infected animals. Laboratory animal models of alveococcosis have produced similar results /7, 8/.

Isoprinosine has also been tested in other laboratory experimental models of pneumocystosis and toxoplasmosis. Its positive effects made it an adjunct agent in cases of HIV infection in order to prevent recurrent P. carinii pneumonia and reactivation of Toxopalma gondii infection.

ABBREVIATIONS USED IN THIS PAPER: None

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Isoprinosine, regardless of its high price is available on the drug market. Its availability as well as the existing clinical evidence encouraged us to focus efforts on achieving better clinical effect in hydatidosis through combined treatment with isoprinosine and albendazole /5/.

This study was aimed at establishing the therapeutic effects of combined treatment with albendazole and isoprinosin in patients with hydatidosis. This is a provisional report.

The following objectives were set:

1. Post surgical, anti-recurrence treatment of patients with lung and liver hydatidosis.
2. Conservative treatment of patients with liver hydatidosis, following PAIR (puncture, aspiration, injection, re-aspiration).
3. Conservative treatment of inoperable liver and lung cysts.
4. Follow-up of the effect.

MATERIALS AND METHODS

The study included 32 adult patients with hydatidosis. Part of them (14) were treated as inpatients, the remaining received outpatient treatment. They were assigned into 3 groups as follows:

1. Patients receiving therapy post surgery - 14 with liver and 2 with lung cysts.
2. Patients treated following PAIR - 4 with liver hydatidosis.
3. 14 patients were treated with albendazole conservatively - 6 with hepatic and 8 with pulmonary cysts.

Four patients were administered Zentel (aldendazole), 28 - Andazol (albendazole) - 200 mg tablets, at a daily dose of 10-15 mg/kg. Treatment course was 30 days with a drug -free intervals of 15 days between courses. Number of courses ranged between 1 to 8. Complete blood count, platelets, serum urea, creatinine, glucose, tramsaminases (ASAT, ALAT) were monitored monthly in the course of treatment.

Serological testing for hydatidosis was performed twice a year. Imaging tests were performed as follows: abdominal US in patients with liver cysts - every 3 months; X-ray in patients with lung cysts - every 6 months. CT was performed in 14 patients before initiation of therapy.

The WHO criteria were used to evaluate treatment outcome. The following rating scale was applied: a) success - reduced cyst dimensions and signs of cyst degeneration; b) partial success - degeneration and minimal reduction of cyst size in some of the cysts, lack of effect in others; c) failure - no visible signs of degeneration.

Isoprinosine, 500 mg tablets, initial daily dosage was 50 mg/kg. For adults it was administered 3 x 2 daily for 8 to 16 days, followed by a maintenance treatment of 2 tablets daily for 25 days regardless of duration of albendazole treatment.

Isoprinosine is an immuno-stimulant that enhances T and B lymphocyte function, NK (natural killers), macrophage and granulocyte function. It restores the immune balance, stimulates response to therapy, prevents secondary infections and improves patients clinical condition. It is safe clinically, only a slight increase in the uric acid level might be observed as an adverse reaction.

RESULTS

1. In our patients, treated with albendazole postoperatively no recurrent or residual cysts were observed during the follow-up (1 to 4 years). Those with liver cysts were subject to abdominal US examination until filling up of the residual cyst cavity, a process which lasted from several months to 1 year (in two cases - more than a year).
2. In patients treated after PAIR (4) a degeneration with filling up of the treated and collapsed hydatid cyst was observed in the first year. They were administered 2 to 4 therapeutic courses with albendazole and isoprinosine according to the number of treated cysts - in two patients with 3 cysts, 4 courses with albendazole were administered.
3. Patients with pulmonary echinococcosis (8) received conservative treatment with albendazole and isoprinosin. In six of them, the cyst content and the residual membranes were eliminated through the sputum. In the remaining two - cyst degeneration was noted on X-ray. Six patients with liver hydatids were treated with albendazole and isoprinosine - degeneration signs in the cysts were noted in them all, including - 4 collapsed cysts (the so-called water lily sign), and 2 cyst with diminished size and significant deformation.

CONCLUSIONS

On the basis of the accumulated evidence it might be concluded that combined treatment with albendazole and isoprinosin produces a more rapid effect (averagely within 6 months). Clinical symptoms (hepatic discomfort, sputum and hemoptoe resolve quickly. Patients recover better following surgery or PAIR. No serious side effects of isoprinosin were noted. Only in 6 patients, a mild elevation of serum uric acid was observed that resolved quickly after discontinuation of the drug. We stress the lack of registered recurrent cysts during the follow-up (1 to 4 years). Due to the limited number of patients, we present only this provisional report. Further cases are under investigation.

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TOWARDS MORE OPTIMAL ANTIMICROBIAL THERAPY OF PYELONEPHRITIS: PERIODIC AUDIT FOLLOWED BY ONE YEAR ANALYSIS

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SUMMARY

An audit of antibiotic prescriptions is a contemporary tool in the assessment of antibiotic policies. The objective of this work is to analyze the antimicrobial treatment of pyelonephritis in 2005 at Nephrology Ward of our hospital following analysis and feedback from 3-month audits undertaken 2003, 2004, and 2005. Appropriateness of treatment was assessed with regard to microbiology results, clinical outcome, adherence to hospital antibiotic policy and national guidelines. Among 106 patients with pyelonephritis, the most frequent pathogens in community acquired uncomplicated pyelonephritis (CAUP) were: Escherichia coli - 67.9%, Klebsiella spp - 11.5%, Enterococci 5.7%. In complicated pyelonephritis (CP) the etiology included: E. coli - 36.7%, Pseudomonas spp - 27.3%, microbial associations of two uropathogens - 31.1%, from: Enterococcus, Enterobacter, Proteus, Klebsiella, Candida spp. Typically, for the treatment of CAUP, I.V. fluoroquinolon (FQ) for 2-10 days (d) was sequenced to P.O. FQ for 0-8 d, - 39.8%; the other choices represented: aminoglycosides - 28.2%; broad-spectrum cephalosporins - 19.2%, amoxicillin/clavulanate - 8.9%, others - 3.9%. For empiric therapy of CP and/or hospital-acquired pyelonephritis (HAP), I.V. FQ or aminoglycosides were prescribed (in absence of specific reasons for another medication). Antimicrobial combinations were used in uro-sepsis or in haemodynamic instability - 4.7 %. While in most cases of CAUP favorable results were obtained, the management of CP and HAP was a challenge due to antibiotic resistance to the first and second line antibiotics and underlying disease. Emergence of ESBL among Enterobacteriaceae (7 patients) and polyresistance in P. aeruginosa, incl. penems (4 patients) was of special concern. The successful treatment needed combined effective antimicrobial therapy and management of abnormalities. In conclusion, the antibiotic therapy of pyelonephritis was evaluated as appropriate, due to good clinical and microbiological results, accepted conclusions from the previous audits, and adherence to the hospital guidelines. The problems seen were related to the emerging antimicrobial resistance (use of reserve and expensive antibiotics) and the managing of complicating conditions.

Key words: pyelonephritis, antibiotic resistance, antibiotic policy, audit

INTRODUCTION

Prudent antibiotic policy is aimed both at better care for patient and limiting the selective antibiotic pressure, the major factor, contributing to development of antibiotic resistance (1, 2, 4, 6, 8-10, 12-16).

In recent years the audit of antibiotic prescriptions was shown to be a useful tool for evaluation of antibiotic treatment courses, as well as an educative measure that promotes further developments in antibiotic policy (3, 5, 7, 11, 21).

An audit of antibiotic prescriptions at Nephrology Department, Medical Institute, Ministry of the Interior, have been undertaken during a 3-month period each year, starting 2003.

The aim of this study is to present the results of the first 2 audits, conducted 2003 and 2004, and then to focus on antibiotic treatment of pyelonephritis during 2005. There were 2 main goals:
- to assess the appropriateness of antimicrobial treatment;
- to study the adherence of medical personnel prescribing antibiotics to the treatment guidelines (12-15, 17-20), and the attitude of prescribers towards different elements in the logistic of choosing appropriate antibiotic.

MATERIALS AND METHODS

A printed form: Control Form for Antibiotic treatment, was developed to be easily filled in by doctors prescribing antibiotics. Patient' information included passport data, age, sex, diagnosis, community/hospital acquired infection, previous antibiotic treatment, co-morbidity, allergy, proofs for infection, severity and elements of emergency. Special attention was paid to the prescriber' attitude: the doctor should describe his motivation to prescribe an antibiotic, and the reasons for his particular choice. The following categories were included: pharmaco-kinetics/pharmaco-dynamics (Pk/Pd) of the antibiotic; - bactericidal, with broad-spectrum, with great therapeutic wideness (low toxicity); - available in the hospital pharmacy, -recommended by National/hospital guidelines; - selected on own experience; - pharmaco-economic considerations; - Microbiology result, incl. susceptibility testing.

Evaluation of the results of treatment was registered in the following categories: in good health, with improvement, no effect, failure, death.

2005 one year analysis of antibiotic treatment of pyelonephritis: the study design represented a retrospective analysis.

Diagnosis was made upon the classical clinical criteria, microbiology and clinical laboratories results, and imaging (at least ultrasound).

Basal patient characteristics included age, sex, co-morbidity (diabetes, obstruction: stone, tumor, catheterization, neurogenic bladder), severity of infection, regimen of antibiotic treatment and clinical and microbiological assessment.

Microbiology methods: quantitative culture of the first morning middle stream urine after toilet; interpretation - according to contemporary criteria; bacterial identification was with API, bioMerieux, France and for Antimicrobial susceptibility testing - the CLSI, USA (NCCLS) criteria, 2002 - were followed.

RESULTS AND DISCUSSION

The 3-month audits of antibiotic prescriptions 2003-2004.

During the first 3-month audit in 2003, antimicrobial treatment in 18 patients was evaluated. The most often prescribed antibiotic regimen for acute pyelonephritis was Ceftriaxone 2 x 2.0 g I.V. x 5 d. Three patients with emergency indications received this therapy. Two other patients (without emergency) received empirically:
- Pefloxacin 2 x 400 mg I.V. x 5 d;
- Co-trimoxazole 2 x 480 mg P.O. x 5 d.

Upon availability of the Microbiological results, it was shown, that one of the patients on Ceftriaxone therapy had susceptible E. coli infection; another one had Enterococcus, and in the last case the antibiotic therapy was not appropriate.

One patient with uro-sepsis (Enterococcus), peritonitis and chronic kidey insufficiency has received Levofloxacin 250 mg, then 125 mg P.O. after a treatment course with Amoxicillin/clavulanic acid.

In a patient with a kidney stone disease, who had treated with Ciprofloxacin 2 x 500 mg P.O. x 10 d, the necessity of antibiotic therapy was not explained and this was considered not-appropriate usage.

During the second audit of antibiotic prescriptions, 2004, 10 patients were evaluated; 7 men, 3 women; average age 57.6 (44-81 y).

The cases were perfectly describes by the physicians with all steps in making decision for antibiotic therapy.

The prescribers' attitude showed a good adherence to the national guidelines and a responsibility in making decision.

Amongst the most common reasons for the prescription of a particular antibiotic were the following: - bactericidal, with a broad-spectrum, with big therapeutic wideness, previous own experience, recommendation by national/hospital guidelines, in vitro susceptibility, the availability in hospital pharmacy.

It became obvious that the care for patients was of good quality, as well as the willing of nephrologists to further improve their knowledge and work.

ABBREVIATIONS USED IN THIS PAPER: None

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2005 analysis of pyelonephritis

The portion of patients with pyelonephritis represented 25.8 % of all hospitalized in the Nephrology department patients - 106 in absolute number and 5.8%, and the total number of days of their hospitalization was 879). Their age -distribution is shown in Fig. 1.

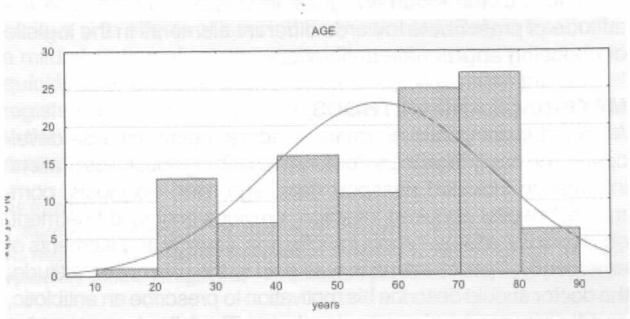

Fig. 1. Patients' age-distribution

From Fig. 2 it can be seen that 37 patients were male and 69 female; as it was expected, the age of the men studied was higher than that of the women. Patients with severe pyelonephritis (Fig. 3) were treated and almost all of them were women - 64.8±13.6 years of age for men and 52.3±19.3 years of age for women.

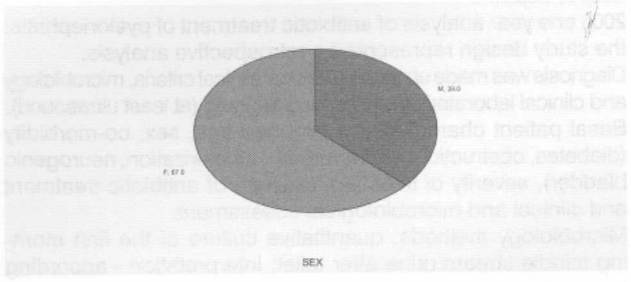

Fig. 2. Patients' sex-distribution

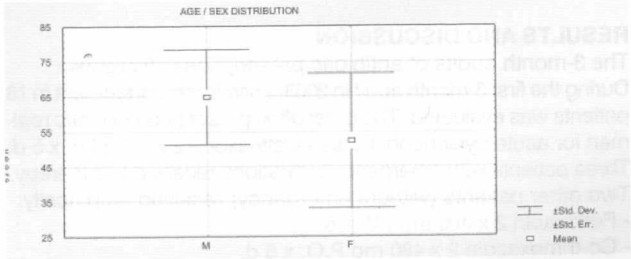

Fig. 3. Patients with severe pyelonephritis: age and sex distribution

Among all of the treated patients - 33 were with diagnosis acute pyelonephritis, 73 - with chronic pyelonephritis (Fig. 4).

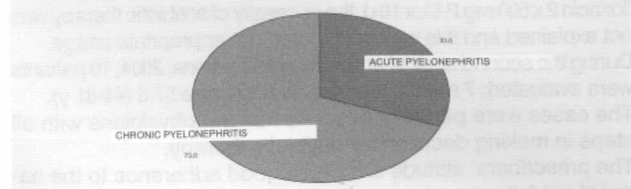

Fig. 4. Relative rate of patients with acute and chronic pyelonephritis

Majority, 75.4% (81 patients) had community acquired uncomplicated pyelonephritis (CAUP), 23.6% (25 patients) had complicated (CP) (kidney or bladder stones with obstruction - 5, catheterization - 6, neurogenic bladder including this with diabetic vegetopathy - 4, immunosuppression - 2) or hospital acquired pyelonephritis (8 patients) (HAP), (Fig. 5).

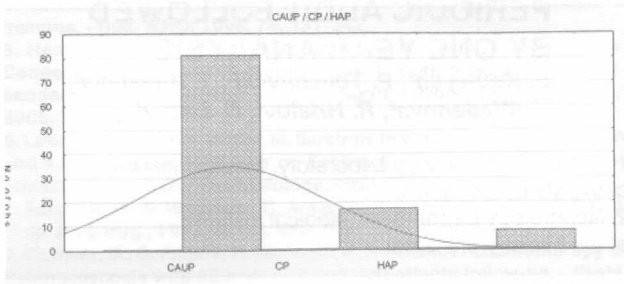

Fig. 5. Distribution of Community acquired- and Hospital acquired- and complicated- pyelonephritis

Microbiology

The commonest bacterial pathogens among patients with community acquired uncomplicated pyelonephritis (CAUP) were: Escherichia coli - 67.9%, followed by, Klebsiella spp - 11.5%, Enterococci 5.7%. In complicated pyelonephritis (CP), including those in catheterized patients, the etiology included: E. coli - 36.7%, Pseudomonas spp - 27.3%, as well as microbial association of two uropathogens - 31.1%, the most frequent from: Enterococcus, Enterobacter, Proteus, Klebsiella, Candida spp. (Table 1).

Antibiotic treatment

Typically, for the treatment of CAUP, a parenteral fluoroquinolon, applied for 2-10 days (d) was then sequenced to oral fluoroquinolon for 0-8 d, which was guided by the symptoms improvement - 47.2%. The other choices represented: aminoglycosides - 24.5%; extended-spectrum cephalosporins - 16.0%, amoxicilline/clavulanate - 9.4%, others - 2.8% (Fig. 6).

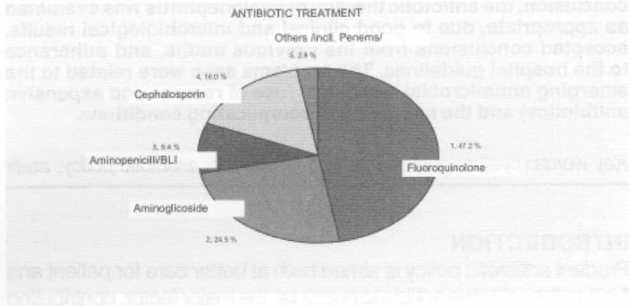

Fig. 6. Antibiotics used in treatment of pyelonephritis, 2005, in pourcent

For initial empiric therapy of CP and/or HAP, the most often parenteral fluoroquinolons or aminoglycosides were prescribed (in absence of specific reasons for another medication). The antimicrobial combinations were used in isolated cases of uro-sepsis or in patients with haemodynamic instability - only 4.7 %.

The duration of the parenteral therapy with fluoroquinolon was shorter, compared to the other antibiotics, because of the sequencing I.V. to per os use (Fig. 7). As it was expected, the prolongation of the parenteral antibiotic therapy was longer in the cases of complicated pyelonephritis (Fig. 8).

While in CAUP favorable microbiological and clinical results were obtained, the management of CP and HAP was a challenge, due to antibiotic resistance to the first and second line antibiotics and to other factors limiting their usage. Emergence of extended spectrum beta-lactamases (ESBL) among Enterobacteriaceae (7 patients) and polyresistance in P. aeruginosa, incl. penems (4 patients) was of special concern. The successful treatment in these cases necessitates a combination with an urological management of the abnormalities.

Table 1. The commonest bacterial pathogens among community acquired and complicated pyelonephritis

	Escherichia coli	Klebsiella spp.	Enterococci	Pseudomonas spp.	Others
CAUP	67.9%	11.5%	5.7%	0	14.9%
CP	36.7%	4.6%	3.5%	27.3%	27.9%

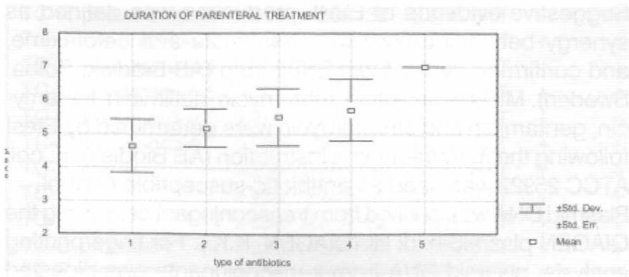

Fig. 7. Duration of antibiotic parenteral therapy of pyelonephritis

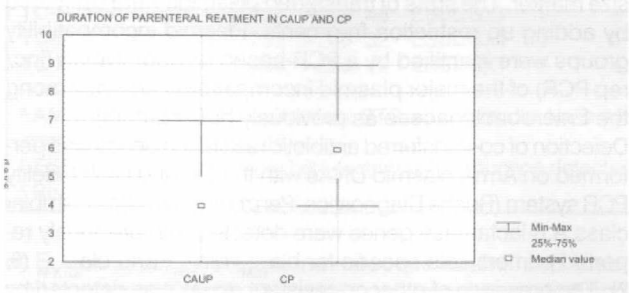

Fig. 8. Duration of treatment in community-acquired and complicated pyelonephritis

CONCLUSIONS

The results from this work showed several important issues:

1. The 3-month audit of antibiotic prescriptions was sufficient to reveal the main problems in antibiotic treatment in the Nephrology department.
2. The well established communications and the common respect between nephrologists, microbiologists and members of the Antibiotic policy committee play a positive role in the amelioration of antibiotic policy.
3. Although the filled in Control forms demonstrated good adherence to national and hospital guidelines, there still remain rooms for improvement:

- some important antimicrobial agents are not available in Bulgaria at present, e.g. nitrofurantoin, fosfomycin, mecillinam, colistin;
- with the emergence and spread of new mechanisms of resistance among uro-pathogens, e.g. ESBLs-producing Enterobacteriaceae and carbapenem-resistant P. aeruginosa, an up-dating of the national guidelines for antibiotic therapy of urinary tract infections is urgent.

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CHARACTERIZATION OF CONJUGATIVE PLASMIDS MEDIATING THE DISSEMINATION OF 16S RIBOSOMAL RNA METHYLASES RESPONSIBLE FOR PANAMINOGLYCOSIDE RESISTANCE OF CLINICAL ENTEROBACTERIACEAE IN A BULGARIAN CANCER HOSPITAL

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SUMMARY

During 2004-2005, high-level resistance to aminoglycosides due to 16S ribosomal RNA methylation was identified in 20 strains of the family Enterobacteriaceae, including Klebsiella pneumoniae, Escherichia coli, Citrobacter freundii, Enterobacter cloacae, Serratia marcescens and Klebsiella oxytoca, at the cancer center of Sofia. The ArmA methylase-mediated aminoglycoside resistance was transferable by conjugation and carried by IncLM plasmids ranging in size from ca. 80.6 to 99.3 kilobase pairs. In addition, all ArmA plasmids carried the following genes: $bla_{CTX-M-3}$ (extended-spectrum beta-lactam resistance), bla_{TEM-1} (ampicillin resistance), ant3"9 (streptomycin-spectinomycin resistance), aac3-II (gentamicin-tobramycin-netilmicin-kanamycin resistance), dfrXII (trimethoprim resistance), sul1 (sulfonamide resistance) and intI1, an integrase associated with class 1 integrons. We conclude that ArmA-mediated panaminoglycoside resistance was disseminated across various species in the family Enterobacteriaceae by closely related, broad-host-range IncLM conjugative plasmids, which conferred similar multidrug resistance phenotypes.

Key words: Enterobacteriaceae, 16S rRNA methylase, conjugative plasmids, PCR-based replicon typing

INTRODUCTION

High-level resistance to aminoglycosides due to 16S ribosomal RNA methylation among various Gram-negative pathogens has been increasingly reported (1). Six 16S rRNA methylase enzymes have been identified: ArmA, RmtA to RmtD and NpmA (1, 2). We recently described the spread of ArmA-mediated aminoglycoside resistance in clinical Enterobacteriaceae isolated in a cancer hospital in Bulgaria (3). The armA genes were located on large plasmids (80-99 kb) that were transferred into Escherichia coli and resulted in multiresistant transconjugants (3). In the present study, we identified the other antibiotic resistance genes carried on these ArmA plasmids and, in order to understand how the genes were disseminated, the ArmA plasmids were identified by a PCR-based replicon typing (inc/rep PCR) of the major plasmid incompatibility groups among the Enterobacteriaceae.

ABBREVIATIONS USED IN THIS PAPER:
ESBL - extended-spectrum beta-lactamase; AMK - amikacin; GEN - gentamicin; STR - streptomycin; SXT - trimethoprim/sulfamethoxazole; PCR - polymerase chain reaction

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MATERIALS AND METHODS

Twenty E. coli ML4909 transconjugants harboring ArmA plasmids described previously were used (3).
Susceptibilities to trimethoprim/sulfamethoxazole, nalidixic acid, tetracycline and chloramphenicol were determined by disc diffusion on Mueller-Hinton agar according to Clinical and Laboratory Standards Institute (CLSI) guidelines (4). Suggestive evidence of ESBL production was defined as synergy between amoxicillin/clavulanate and cefotaxime, and confirmed by the Etest ESBL strip (AB Biodisk, Solna, Sweden). MICs of amikacin, tobramycin, netilmicin, kanamycin, gentamicin and streptomycin were determined by Etest following the manufacturer's instruction (AB Biodisk). E. coli ATCC 25922 was used as antibiotic-susceptible control.
Plasmid DNA was purified from transconjugant cells using the QIAGEN plasmid-midi kit (QIAGEN, K.K.). For fingerprinting analysis, plasmid DNA from transconjugants was digested with either EcoRI or SacI endonucleases (Toyobo, Co., Ltd, Osaka, Japan) and subjected to electrophoresis on 0.7% agarose gel at 110 V for 2 h. Lambda DNA digested with EcoT14I (Takara Bio Inc., Otsu, Japan) was used as a DNA size marker. The sizes of transferred plasmids were estimated by adding up restriction fragments. Plasmid incompatibility groups were identified by a PCR-based replicon typing (inc/rep PCR) of the major plasmid incompatibility groups among the Enterobacteriaceae as previously described (5).
Detection of co-transferred antibiotic resistance genes was performed on ArmA plasmid DNAs with the Expand High Fidelity PCR system (Roche Diagnostics, Penzberg, Germany). Ambler class A β-lactamase genes were detected with previously reported primers sets specific for bla_{TEM}, bla_{SHV}, and bla_{CTX-M} (6, 7). The presence of other co-resistant genes was detected by PCR using primers as previously described (8). Amplification of DNA was performed in a GeneAmp 9600 thermal cycler (Perkin-Elmer, Norwalk, CT). Amplicons were purified with a QIAquick PCR purification kit (QIAGEN, K.K., Tokyo, Japan) and sequenced on both strands using a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Sequence analyses and comparison with known sequences were performed with the BLAST programs at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST).

RESULTS AND DISCUSSION

All transconjugants harboring ArmA plasmids were highly resistant (MICs, >256 μg/ml) to 4,6-disubstituted deoxystreptamines such as amikacin, tobramycin, netilmicin, kanamycin and gentamicin, which was consistent with the presence of the armA methylase gene. In addition, all transconjugants were resistant to trimethoprim-sulfamethoxazole and showed ESBL phenotype by the double-disk synergy test (Table 1).
PCR for bla_{CTX-M} and subsequently for $bla_{CTX-M-1}$-like genes yielded an amplicon, which was confirmed to be $bla_{CTX-M-3}$ after sequencing. In addition, the bla_{TEM-1} was also identified in all armA-carrying plasmids. PCR analyses revealed also the presence of ant3"9 (streptomycin-spectinomycin resistance), aac3-II (gentamicin-tobramycin-netilmicin-kanamycin resistance), dfrXII (trimethoprim resistance), sul1 (sulfonamide resistance) and intI1, an integrase associated with class 1 integrons. All the resistance genes were always carried by IncL/M plasmids ranging in size from ca. 80.6 to 99.3 kb (Table 1).
The 20 ArmA plasmids were restricted with EcoRI or SacI endonucleases, which resulted in four distinct patterns (Fig. 1). The fragment patterns of conjugative plasmids were very similar, and differences were restricted to the mobility of three fragments. On the basis of these differences, the plasmids were assigned to four types A, B, C and D. As shown in Table 1, type A plasmid was predominant and present in all transconjugants except the transconjugants

Table 1. Characteristics of E. coli transconjugants harboring ArmA plasmids

Transconjugant	Etest MIC (μg/ml)[a]			Associated resistance patterns[b]	Results of PCR and sequencing							Plasmid size(kb)	Plasmid type
	AMK	GEN	STR		IncL/M	$bla_{CTX-M-3}$	bla_{TEM-1}	ant3"9	aac(3)-II	dfrXII	sul1		
E. coli ML4909[c]	2	1	8	-	-	-	-	-	-	-	-	-	-
TC-K. pneumoniae 1	>256	>256	32	ESBL SXT	+	+	+	+	+	+	+	90.2	A
TC-K. pneumoniae 2	>256	>256	32	ESBL SXT	+	+	+	+	+	+	+	90.2	A
TC-K. pneumoniae 3	>256	>256	32	ESBL SXT	+	+	+	+	+	+	+	90.2	A
TC-K. pneumoniae 4	>256	>256	32	ESBL SXT	+	+	+	+	+	+	+	90.2	A
TC-K. pneumoniae 5	>256	>256	32	ESBL SXT	+	+	+	+	+	+	+	90.2	A
TC-K. pneumoniae 6	>256	>256	32	ESBL SXT	+	+	+	+	+	+	+	90.2	A
TC-K. pneumoniae 7	>256	>256	32	ESBL SXT	+	+	+	+	+	+	+	90.2	A
TC-E. coli 1	>256	>256	32	ESBL SXT	+	+	+	+	+	+	+	90.2	A
TC-E. coli 2	>256	>256	32	ESBL SXT	+	+	+	+	+	+	+	90.2	A
TC-E. coli 3	>256	>256	32	ESBL SXT	+	+	+	+	+	+	+	99.3	B
TC-E. cloacae 1	>256	>256	32	ESBL SXT	+	+	+	+	+	+	+	90.2	A
TC-E. cloacae 2	>256	>256	32	ESBL SXT	+	+	+	+	+	+	+	90.2	A
TC-E. cloacae 3	>256	>256	32	ESBL SXT	+	+	+	+	+	+	+	90.2	A
TC-C. freundii 1	>256	>256	48	ESBL SXT	+	+	+	+	+	+	+	90.2	A
TC-C. freundii 2	>256	>256	16	ESBL SXT	+	+	+	+	+	+	+	90.2	A
TC-C. freundii 3	>256	>256	32	ESBL SXT	+	+	+	+	+	+	+	90.2	A
TC-S. marcescens 1	>256	>256	32	ESBL SXT	+	+	+	+	+	+	+	80.6	C
TC-S. marcescens 2	>256	>256	48	ESBL SXT	+	+	+	+	+	+	+	80.6	C
TC-S. marcescens 3	>256	>256	32	ESBL SXT	+	+	+	+	+	+	+	94.0	D
TC-K. oxytoca	>256	>256	48	ESBL SXT	+	+	+	+	+	+	+	94.0	D

[a] AMK, amikacin; GEN, gentamicin; STR, streptomycin; kanamycin; MIC values of tobramycin, netilmicin and kanamycin for all transconjugats were >256 μg/ml.
[b] ESBL, extended-spectrum beta-lactamase production detected by double-disk synergy test; SXT, trimethoprim/sulfamethoxazole.
[c] Recipient cells

of E. coli strain 3, Serratia marcescens strains and Klebsiella oxytoca. Notably, Klebsiella pneumoniae strain 5 and Enterobacter cloacae strain 1, K. pneumoniae strain 7 and E. coli strain 1, and S. marcescens strain 3 and K. oxytoca, possessing couple common plasmid type, were isolated each couple from a single patient specimen (3). These three cases suggested that in vivo horizontal transfer of the ArmA determinant had possibly occurred.

The dissemination mechanisms of the genes that encode 16S rRNA methylases are of clinical significance since the genes confer a high-level of resistance to all clinically available aminoglycosides, except streptomycin, and they were often linked to other resistance determinants such as bla_{TEM-1}, bla_{CTX-M}, sul1 and dfrXII (8, 9, 10). The exploration of genetic environments of armA genes revealed that armA was part of functional composite transposon Tn1548 in the plasmid pIP1204 (9), suggesting that the spread of armA resulted from transposition. Another mechanism, the dissemination of armA by conjugative plasmids, has been demonstrated in a few studies: armA by a broad-host-range IncL/M conjugative plasmids (9, 11) and by a self-transferable IncN plasmid in an E. coli pig isolate (12).

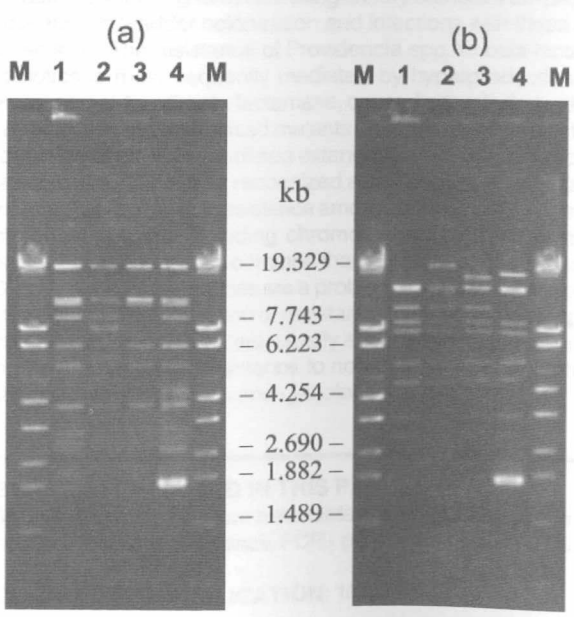

Fig. 1. (a) EcoRI and (b) SacI fingerprints of conjugative plasmids for armA-positive Enterobacteriaceae isolates. Lanes: M, EcoT14I-digested lambda DNA marker; 1, plasmid of type A; 2, type B; 3, type C; 4, type D

Our results indicated that armA was disseminated among enterobacteria from six species by similar, broad-host-range IncL/M conjugative plasmids. It was linked to bla_{TEM-1} and $bla_{CTX-M-3}$, which confer resistance to all beta-lactams except carbapenems and cephamycins, and also to streptomycin-spectinomycin, trimethoprim, and sulfonamides resistance determinants. These data support previous findings that armA correlates frequently with CTX-M extended-spectrum beta-lactamase genes contributing to multiresistant phenotypes (8, 9, 10, 11).

CONCLUSIONS

The present study demonstrated that ArmA-mediated panaminoglycoside resistance was disseminated across various species in the family Enterobacteriaceae by closely related, broad-host-range IncLM conjugative plasmids, which conferred similar multidrug resistance phenotypes. The spread of such multidrug resistance plasmids among bacterial pathogens has a potential impact on the empirical management of complicated infections that may be treated initially with cephalosporins and aminoglycosides.

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PLASMID-MEDIATED PER-1 EXTENDED-SPECTRUM BETA-LACTAMASE IN PROVIDENCIA RETTGERI FROM BULGARIA

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SUMMARY

A multiresistant Providencia rettgeri strain was isolated from the urine of a 38-year-old female cancer patient during an episode of febrile neutropenia that followed a course of postoperative adjuvant chemotherapy. The strain was resistant to beta-lactams (except cephamycins and carbapenems), aminoglycosides, fluoroquinolones, chloramphenicol, tetracycline, trimethoprim and sulfonamides. Clavulanic acid restored activity of oxyimino-cephalosporins, suggesting the production of an extended-spectrum beta-lactamase (ESBL). PCR and sequencing with primers specific for known ESBL genes identified blaPER-1. The PER-1 determinant was transferable by conjugation and cotransferred with blaTEM-1 (ampicillin resistance), aac(6')-Ib (amikacin-tobramycin-kanamycin resistance), gentamicin, chloramphenicol, trimethoprim and sulfonamide resistance determinants. Plasmid-mediated quinolone resistance determinants (qnr and aac(6')-Ib-cr) were not detected. This is the first description of plasmid-mediated PER-1 enzyme in Enterobacteriaceae in Bulgaria. Since PER-1-positive Gram-negative pathogens have been increasingly isolated in Turkey and Europe, and given the potential for further dissemination, nationwide early recognition and rapid identification of PER-1-producing bacteria should be considered to avoid further spread of this resistance determinant.

Key words: Providencia rettgeri, extended-spectrum beta-lactamase, PER-1

INTRODUCTION

The genus Providencia is a member of the tribe Proteeae and consists of five species, four of which, P. alcalifaciens, P. stuartii, P. rettgeri and P. rustigianii, are known to occur in clinical specimens. Providencia infections are almost exclusively nosocomial and patients with long-term indwelling urinary catheters are prone to developing bladder colonization and infections with these organisms (1). The resistance of Providencia spp. to beta-lactam antibiotics is most frequently mediated by hyperproduction of chromosomal AmpC beta-lactamase, caused either by induction or by selection of derepressed mutants (2). In the last decade, the production of plasmid-mediated extended-spectrum beta-lactamases (ESBLs) has been recognized as an additional important emerging mechanism of resistance among members of the family Enterobacteriaceae, including chromosomal AmpC-producing species (3). Although less common than AmpC hyperproduction, ESBLs among these species are a problem of great concern due to the potential transmission of resistance to other bacterial species and because ESBLs are usually encoded by plasmids that also harbor genes for resistance to non-beta-lactam antibiotics such as aminoglycosides and quinolones (4, 5).

ABBREVIATIONS USED IN THIS PAPER:
ESBL - extended-spectrum beta-lactamase; PMQR - plasmid-mediated quinolone resistance; PCR - polymerase chain reaction

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Here, we report the identification of plasmid-mediated PER-1 ESBL in multiresistant P. rettgeri strain isolated from cancer patient in Bulgaria.

MATERIALS AND METHODS

P. rettgeri 20836 was isolated from the urine of a 38-year-old female cancer patient, during an episode of febrile neutropenia that followed a course of postoperative adjuvant chemotherapy, at the oncology hospital of Sofia in 1997. It was identified by standard biochemical tests and confirmed with an automated identification system (Vitek AMS; bioMerieux Vitek Systems Inc., Hazelwood, MO).

Susceptibility to antimicrobials was initially determined by disc diffusion on Mueller-Hinton agar according to Clinical and Laboratory Standards Institute (CLSI) guidelines (6). Suggestive evidence of ESBL production was defined as synergy between amoxicillin/clavulanate and at least one of the following antibiotics: cefotaxime, ceftazidime, aztreonam or cefepime. MICs were determined by a broth microdilution method using Vitek system (Vitek AMS; bioMerieux). Susceptibility interpretations were defined according to CLSI-2007 breakpoints (7). Escherichia coli ATCC 25922 was used as an antibiotic-susceptible control.

Conjugation experiments were carry out by direct mating and filter mating methods using rifampicin-resistant recipient E. coli J53. Transconjugants were selected on brain heart infusion agar supplemented with rifampicin (300 mg/L) and ceftazidime (10 mg/L).

PCR screening for genes encoding ESBLs was performed on boiled cell lysate of clinical strain and its transconjugant with the Expand High Fidelity PCR system (Roche Diagnostics, Penzberg, Germany). The bla_{PER}, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{VEB} and bla_{GES} genes were amplified using specific primers and reaction conditions as previously described (8). As plasmid-mediated quinolone resistance (PMQR) determinants (qnr and aac(6')-Ib-cr) have been found with ESBL genes, PCR screening for qnr and aac(6')-Ib-cr genes was performed as previously described (9, 10). Amplification of DNA was performed in a GeneAmp 9600 thermal cycler (Perkin-Elmer, Norwalk, CT). Amplicons were purified with a QIAquick PCR purification kit (QIAGEN, K.K., Tokyo, Japan) and sequenced with the CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA). Sequence analyses and comparison with known sequences were performed with the BLAST programs at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST).

RESULTS AND DISCUSSION

A routine susceptibility test on P. rettgeri 20836 revealed marked synergy between oxyimino-cephalosporin discs and clavulanate disc, indicating ESBL production. In addition, the strain was resistant to aminoglycosides, fluoroquinolones, chloramphenicol, tetracycline, trimethoprim and sulfonamides, but it remained susceptible to cephamycins and carbapenems (Table 1).

PCR with primers specific for known ESBL genes yielded amplicons for bla_{PER} and bla_{TEM}, identified as PER-1 and TEM-1, respectively, using sequencing. Ceftazidime resistance marker was successfully transferred by conjugation to E. coli J53 from P. rettgeri 20836. The presence of blaPER-1 gene in E. coli transconjugant was confirmed by PCR analysis, indicating its location on transferable plasmid. Susceptibility testing and PCR analysis revealed that PER-1 determinant was cotransferred with aac(6')-Ib (amikacin-tobramycin-kanamycin resistance), gentamicin, chloramphenicol, trimethoprim and sulfonamide resistance determinants (Table 1). Plasmid-mediated quinolone resistance determinants (qnr and aac(6')-Ib-cr) were not detected.

PER-1 ESBL was first identified in 1993 in Pseudomonas aeruginosa isolate from a Turkish patient in France (11). Since then, blaPER-1 gene has been increasingly detected in P. aeruginosa and Acinetobacter baumannii isolates from Turkey and many European countries, but still rarely in Enterobacteriaceae (12). Interestingly, regarding enterobacteria, PER-1 enzyme has marked preference toward Salmonella isolates and organisms from tribe Proteae, such as Proteus mirabilis, P. stuartii and P. rettgeri (12, 13). The fact that since 1997, when the reported here PER-1-positive P. rettgeri 20836 has been isolated, two more patients infected with PER-1-prodicing P. rettgeri strans

Table 1. Antimicrobial susceptibilities of PER-1-producing P. rettgeri 20836, its transconjugant and the E. coli J53 recipient

Strain	β-lactamases	MICs of β-lactams (mg/L)[a]						Other resistances[b]
		CAZ	CTX	ATM	FEP	FOX	IPM	
P. rettgeri 20836	PER-1/TEM-1	>=32	8	16	8	<=2	<=4	Ak Cm Cp Gm Km Nt Nx Su Tc Tm Tp
Transconjugant	PER-1/TEM-1	>=32	8	16	8	<=2	<=4	Ak Cm Cp Gm Km Nt Nx Su Tm Tp
E. coli J53	-	<=8	<=4	<=8	<=4	<=2	<=4	-

[a] CAZ, ceftazidime; CTX, cefotaxime; ATM, aztreonam; FEP, cefepime; FOX, cefoxitin; IPM, imipenem.
[b] Resistance abbreviations: Ak, amikacin; Cm, chloramphenicol; Cp, ciprofloxacin; Gm, gentamicin; Km, kanamycin; Nt, netilmicin; Nx, nalidixic acid; Su, sulfonamides; Tc, tetracycline; Tm, tobramycin, Tp, trimethoprim.

were recovered in 1999 and 2000, respectively, and that a small outbreak of PER-1-prodicing A. baumannii has been registered in 2001 at the same hospital, may indicate that the blaPER-1 gene has spread in Gram-negative pathogens in Bulgaria.

CONCLISIONS

This is the first description of plasmid-mediated PER-1 enzyme in Enterobacteriaceae in Bulgaria. Since PER-1-positive Gram-negative pathogens have been increasingly isolated in Turkey and Europe, and given the potential for further dissemination, nationwide early recognition and rapid identification of PER-1-producing bacteria should be considered to avoid further spread of this resistance determinant.

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CLINICAL THERAPY AND CHEMOPROPHYLAXIS OF TRICHINOSIS WITH BENZIMIDAZOLES

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SUMMARY

Etiological treatment of trichinosis was possible in the last 2-3 decades due to the synthesis and administration of benzimidazole derivatives. Thiabendazole, mebendazole, flubendazole and albendazole were introduced consecutively in clincal practice. Accumulated evidence proved that medendazole and flubendazole are effective in the intestinal stage of trichinosis. Albendazole is effective during intestinal infection and to some extent during the tissue stage. Thiabendazole is no longer used in clinical practice due to its significant adverse reactions. Early diagnosis of trichinosis and epidemiological surveillance identifies patients at risk. Adequate chemoprophylaxis prevents further expansion of the outbreak.

Key words: trichinosis, benzimidazol

INTRODUCTION

In the second half of 19th century, after the discovery of Trichinella spiralis as human pathogen, unsuccessful attempts have been made at its etiological treatment. Various well-known chemical substances (carbolic acid, benzene, thymol, gentian violet, arsenic, mercury) as well as natural products (Alium sativum) have been used (9). In Bulgaria, the doyen of Bulgarian pharmacology, P. Nikolov applied Atebrin and Emetinum hydrocloridum to treat human infection with Trichinella spiralis. Later, Diethyl-carbamazine (DEC, Loxuran) was used as etiological treatment of trichinosis.
In 1960s, after the introduction of thiabendazole (the first benzimidazole derivative), therapeutic options of trichinosis expanded. Initial observations (W. Campbell, 1961) showed that benzimidazole molecules exhibit a parasitocidal effect on T. spiralis (8, 9). Since that time, the action of various synthetic benzimidazole derivatives has been tested in vitro and in vivo either on isolated Trichinella larvae or in animal models. However, only a few of these agents have been an object of systematic clinical and experimental studies (thiabendazole, cambendazole, DEC - D. Georgieva, 1972 and thiabendazole - V. Radoev et all., 1972-1975) (8, 9).

AIM

The aim of this study is to present our clinical observations on the therapeutic effect of the benzimodazole compounds (thiabendazole, medendazole, flubendazole and albendazole) on patients with trichinosis. Literature review of chemotherapy and chemoprophylaxis of trichinellosis is presented as well.
The pharmacological characteristics of benzimidazole derivatives are as follows (9):
1. They are effective against the immature and sexually mature form of intestinal trichinae (the effect is more pronounced against the immature forms).
2. They suppress larval development at a small, non-lethal for the adult forms dosage.
3. They are active against the migrating, capsule forming and capsulated parasites. In these cases, higher dosage and prolonged treatment courses are usually necessary.
4. Anti-parasitic effects are due to cellular micro-tubular blockage as a result of inhibited tubular polymerization. Some derivatives inhibit cellular glucose uptake.
5. Unlike other antihelmintics, benzimidazole derivatives are recognized as etiological treatment of trichinosis in all stages of infection. Some were designed especially for that - Vermox forte (mebendazole, tabl. 500 mg) and Escazol (albendazole, tabl. 400 mg).
There is data that different trichina types, sub-types and geographical strains react differently to the action of benzimidazoles. However, there differences are mainly quantitative, not qualitative.

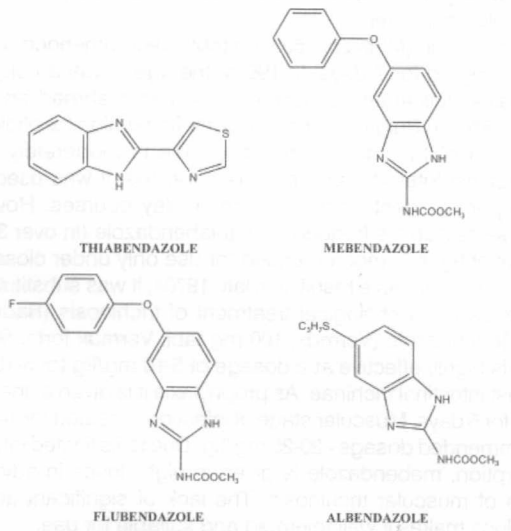

Fig. 1. Chemical structure of benzimodazole drugs, used in the etiological treatment of trichinosis

MATERIALS AND DISCUSSION

There is no universal treatment protocol for trichinosis. As mentioned above, benzimidazole derivatives have a direct parasitocidal effect on intestinal larvae in all stages, as well as on migrating, encapsulating and capsulated muscular trichina larvae (3, 4). Early treatment destroys immature intestinal parasites. At a later stage, these agents inhibit the reproductive capacity of mature parasites and in higher doses, kill them. During parasitic migration, treatment blocks muscular invasion by the parasites or at least reduces it significantly. Later in the course of infection, the larvocidal effect of benzimidazoles is limited but is still present. Examination of histological specimens shows inflammatory response in the muscles, at the site of parasitic invasion leading to destruction of capsulated parasites. In Bulgaria, muscle biopsy is not a widely used diagnostic technique, but in the US and Japan it is crucial for etiological diagnosis. In our opinion, muscle biopsy is indicated in cases with chronic polymyositis (for differential diagnosis purposes) and to follow up treatment response. Chronic polymyositis is likely to develop as a sequel of trichina infection, especially in moderate and severe forms of infection (5).
In an animal model, we established a significant larvocidal effect in trichina infected guinea pigs, treated with some novel derivatives of benzimidazoles. In these cases, a degeneration of capsulated muscular parasites was observed, following a 10 day treatment course (1).
Therapeutic effect of benzimidazole compounds is well established in cases of large outbreaks of trichinosis. There is data in the literature, that muscle biopsy of infected patients documented lack of viable muscular trichina larvae following treatment with thiabendazole. Muscle biopsy was performed in 20 patients. Only two of them had viable muscular parasites post treatment (8).
When treatment with benzimidazoles is initiated in the acute stage of trichinosis, within a few days the fever subsides, edema and muscle pains gradually resolve. If patients receive treatment in the intestinal stage of infection, it would prevent development of clinical

ABBREVIATIONS USED IN THIS PAPER: None

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diseases. This fact gives the grounds to consider that benzimidazoles are indicated as prophylactic treatment in the incubation period in all patients having a history of ingesting contaminated meat products (4). In late trichinosis, some authors consider that benzimidazole therapy is contraindicated as destruction of capsulated muscle larvae leads to chronic myositis. So far no optimal treatment period has been established. However, based on clinical experience, most authors recommend treatment of at least two week duration in all stages and all forms of trichinosis. Rarely if there is clinical and laboratory data of chonic myiositis (EMG etc.) repeated courses with benzimidazoles are recommended (9).

Drug agents for etiological treatment of trichinosis. The most widely used benzmidazole derivatives are presented in chronological order.

Thiabendazole (Mintezol), 500 mg tabl. - recommended dosage - 25-50 mg/kg for 7 days. In 1960s, the agent was an object of extensive clinical and experimental research abroad and later (1971-1976) in Bulgaria. It provided the first option for etiological treatment of trichinosis. Thiabendazole is moderately effective against intestinal and muscle parasites. It was used as a prophylactic agent as well - in three day courses. However, adverse reaction is frequent with thiabendazole (in over 30% of the patients). It is recommended for use only under close clinical observation. As a result, it in late 1970s, it was substituted for mebendazol as etiological treatment of trichinosis (Radoev et all.) Medendazole (Vermox, 100 mg tabl. Vermox forte, 500 mg tabl.) is highly effective at a dosage of 5-10 mg/kg for 5-10 days against intestinal trichinae. As prophylaxis it is given at the same dose for 5 days. Muscular stage of infection is treated for 14 days, recommended dosage - 20-25 mg/kg. Due to its limited intestinal absorption, mebendazole is given in high doses in advanced forms of muscular trichinosis. The lack of significant adverse reactions makes it well-tolerated and suitable for use.

Albendazole (Zentel, Andazol, Escazol), 200 (400) mg tabl. is highly effective against intestinal trichinae at a dosage of 10-15 mg/kg (intestinal absorption up to 100% have been established in experimental animal models). It is recommended as prophylaxis in patients, who have ingested contaminated meat. In mild forms of disease, the drug is administered at a dose of 10-15 mg/kg for 7 -10 days. In moderate and severe forms, treatment is prolonged up to 10-14 days. In the last decade, systematic research of the drug agent involved more than 100 patients with trichinosis. Chemoprophylaxis was administered to more than 150 patients. None of them developed symptoms of the disease.

All drug agents, described above are contraindicated in pregnant women. Among benzimidazole derivatives only flubendazole can be administered to pregnant women (9).

Flubendazole (Fluvermal, 100 mg tabl.) is similar in its action to mebendazole. So far, research shows that it has no teratogenic effect and can be given to pregnant women. Dosage and treatment regimens are similar to that of mebendazole. Experimental and clinical trials have documented its high efficiency and safety. Adverse reactions are uncommon. It is especially good for mass chemo prophylaxis to all patients who at risk of developing the disease after consumption of contaminated meat.

It is recommended that all patients with mild to moderate diseases receive hospital treatment. Mild forms can de treated on an outpatient basis. They require close surveillance and follow-up by a GP and consultant-parasitologist. Mass destruction of larvae after initiation of treatment is likely to result in worsening of muscle pains, edema, fever and white blood cell and eosinophil counts. For this reason, treatment is usually initiated at lower doses (from 1/3 of the therapeutic doses) for 2-3 days. Optimal dosage is achieved afterwards. Repeated treatment courses are indicated rarely within three months in cases of suspected recurrent infection due to viable adult parasites in the intestinal tract. Such treatment is also indicated in cases of prolonged myalgias with EMG data of myositis and high eosinophil count in the convalescence period. Short courses with corticosteroids are indicated and they influence well the clinical picture. However, due to the risk of development of chronic myositis they should be discontinued promptly. Muscle biopsy is especially important in cases with chronic myositis.

On the basis of accumulated evidence it might be concluded that:

1. Trichinosis is a curable disease. Benzimidazole derivatives provide highly effective and etiological treatment. In order to adjust dosage, careful clinical evaluation of disease severity is required the disease form should be determined - asymptomatic trichinosis, mild, moderate, severe or complicated disease.
2. The following agent can be used as ethiological treatment:

a) for prophylaxis:

- Mebendazole (Vermox) - 100 mg tid for 5 days in all ages, contraindicated in pregnant women.
- Flubendazole (Fluvermal) - 100 mg twice daily for 5 days, in all age groups, safe usage in pregnant women Albendasole (Zentel) - 200 mg twice daily for 3 days, contraindicated in pregnant women.

b) treatment regimens:

- Albendazole (Zentel) - 10-15 mg/ kg daily with a gradual increase of dosage until optimal dosage is achieved. Treatment duration - mild trichinosis - 5-7 10 days, moderate and severe diseases - 10-14 days.
- Mebendazole (Vermox) and flubendazole (Flumerval), 20-25 mg/ kg, 5 mg/kg in children. Treatment is initiated at 1/3 of the prescribed dosage for the first three days. There is a gradual increase in dosage until optimal is achieved. Treatment duration - 7-10 days in mild diseases, 14 - in moderate and severe disease.

3. Follow-up of all patients is mandatory due to the presence of prolonged convalescence and persistent clinical and laboratory data of disease. Rehabilitation is recommended if necessary.

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