Indexed by
SCOPUS
from 2008

PROBLEMS
of Infectious and Parasitic Diseases

NATIONAL CENTER OF INFECTIOUS AND PARASITIC DISEASES
SOFIA, VOLUME 44, NUMBER 1/2016

ISSN 0204-9155

1504 Sofia; 26, Yanko Sakazov Blvd.
Tel.: +359 2/ 846 83 07, Fax: +359 2/ 943 30 75
e-mail: infovita@ncipd.org

PROBLEMS OF INFECTIOUS AND PARASITIC DISEASES
VOLUME 44, NUMBER 1/2016

Preprinting and Printing by Maker Arts & Alliance print

CONTENTS

Instructions to Authors

Papers should not have been previously published or be currently under consideration for publication.

Manuscripts must be written in English, using British spelling. All manuscripts should be single-spaced, with wide margins and numbered pages. MS Word should be used for word processing, 12-point Times New Roman font.

Named authors must fit the following three criteria:

1. Substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data;
2. Drafting the article or revising it critically for important intellectual content; and
3. Final approval of the version to be published.

All people who meet the three criteria should be named as authors. Those who participate in the study but do not meet the requirements of authorship should be acknowledged as contributors.

TITLE PAGE

The title page must contain: 1) title, name and surname of the authors; 2) names of the institution(s) where the research was carried out; 3) the name and full postal address, e-mail address and telephone numbers of the corresponding author; 4) three to five key words.

ABSTRACT

The abstract should contain about 250 words and must be structured as follows: background, material and methods, results, conclusions. Review Articles should have an informative, unstructured abstract of about 250 words. Brief reports should have a short abstract of no more than 150 words.

TEXT

The text should contain introduction, material and methods, results, discussion and references. No particular format is required for review articles.

ACKNOWLEDGEMENTS

Individuals who supplied facilities, strains or reagents, or gave advice may be acknowledged. Also, supporting grants may be mentioned.

REFERENCES

References should be numbered in order of appearance in the text, in parenthesis, not superscripts, as shown bellow:

Journal articles:

Vellinga A, Cormican M, Hanahoe B, Murphy AW. *Predictive value of antimicrobial susceptibility from previous urinary tract infection in the treatment of re-infection*. Br J Gen Pract. 2010; 60(576):511-513.

Books:

Rosa PA, Hogan D, Margolis N. *Molecular analysis of the major outer surface protein locus from a divergent Borrelia burgdorferi isolate from Europe*. In: Schutzer SE. Lyme borreliosis: Molecular and immunologic aspects. Cold Spring Harbor Laboratory Press, 1992, 95-110.

TABLES

Tables should be incorporated in the manuscript file, not as separate files, MS Word table tool, no wider than 17 cm.

FIGURES

Figures should be provided as separate files, not embedded in MS Word, PC file formats (e.g., MS Excel/PowerPoint). Image files should be submitted without text content as high-resolution (300 dpi/ppi minimum) TIFF or JPG files.

INFORMED CONSENT

Identifying details of patients should be omitted. Identifying information, including patients' names, initials, or hospital numbers, should not be published unless the the patient (or parent or guardian) gives written informed consent for publication. When informed consent has been obtained it should be indicated in the published article.

PHENOTYPIC DETECTION OF 16S RRNA METHYLTRANSFERASE-PRODUCING *ENTEROBACTERIACEAE* BY ROUTINE ANTIBIOGRAM

S. Sabtcheva

Laboratory for Clinical Microbiology, Specialised hospital for active treatment in oncology, Sofia, Bulgaria

ABSTRACT

16S rRNA methyltransferase (16S-RMTase)-mediated aminoglycoside resistance, formerly confined to aminoglycoside producers as a mechanism of self-defence, has recently been identified in Gram-negative pathogens. The genes encoding these determinants are usually associated with extended-spectrum β-lactamases (ESBLs), but recently they have been found in association with carbapenemases resulting in difficult-to-treat multidrug-resistant bacteria. In this study, we analysed resistance phenotypes of 120 well-characterised, consecutive 16S-RMTase-producing *Enterobacteriaceae*, collected at a cancer hospital during an 11-years survey period (2004-2015). Our results suggest that concomitant use of gentamicin, amikacin, and apramycin provide a reliable screening for 16S-RMTase production in all *Enterobacteriaceae*. Moreover, the addition of meropenem as an indicator of carbapenemase production and the double-disc synergy test with cefepime for ESBL detection provided a good tool for further surveillance of 16S-RMTase-producing *Enterobacteriaceae* at the hospital level. In conclusion, concomitant high level resistance to gentamicin and amikacin, but susceptibility to apramycin could be a sensitive screening tool for rapid detection, improved surveillance, and appropriate treatment of 16S-RMTase-producing multidrug-resistant enterobacterial pathogens.

ADDRESS FOR CORRESPONDENCE:
Stefana Sabtcheva
Laboratory for Clinical Microbiology
Specialised Hospital for Active Treatment in Oncology
6 Plovdivsko pole Str
1756 Sofia, Bulgaria
Tel. 8076293
E-mail: stefanasabtcheva@gmail.com

Keywords: *Enterobacteriaceae*, aminoglycoside resistance, 16S rRNA methyltransferase

INTRODUCTION

Aminoglycoside along with β-lactam resistance raises clinical concerns and its occurrence could compromise the widely used combinations of aminoglycoside and b-lactam antibiotics for the treatment of serious infections caused by pathogenic bacteria. Although the inactivation by aminoglycoside-modifying enzymes remains the most prevalent and clinically relevant mechanism of aminoglycoside resistance, since 2003 an emerging mechanism - the alteration of ribosomal targets by methylation, has gradually increased (1). Unlike aminoglycoside-modifying enzymes that vary in their substrate ranges, the acquired 16S rRNA methyltransferase (16S-RMTase) confers high-level resistance to all 4,6-disubstituted 2-deoxystreptamine (gentamicin, sisomicin, isepamicin, kanamycin, amikacin, tobramycin, and arbekacin), but not to 4,5-disubstituted 2-deoxystreptamine (neomycin), streptomycin, and apramycin (1, 2).

Taking into account the peculiar enzymatic function of 16S-RMTases and the resulting extraordinary high-level resistance to all clinically useful aminoglycosides, we attempted to develop a cost-effective disc-based method to screen for 16S-RMTase producers within the routine antibiogram.

MATERIALS AND METHODS

From January 2004 to December 2015 all *Enterobacteriaceae* isolated at a 242-bed cancer hospital in Sofia were systematically tested against gentamicin, amikacin, tobramycin, netilmicin, kanamycin and apramycin with disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Isolates that exhibited concomitantly no inhibitory zone around all aminoglycosides tested, except apramycin, had already been confirmed by PCR analysis to harbour known16S-RMTase genes (3, 4, 5). The so created collection of well-characterised, consecutive 16S-RMTase-producing *Enterobacteriaceae* was analysed in an attempt to select the best phenotypic indicators able to detect the 16S-RMTase producers within routine susceptibility testing at the hospital.

RESULTS

Between January 2004 and December 2015, routinely performed disc diffusion susceptibility tests composed of the above mentioned aminoglycosides revealed the presence of a resistance

phenotype compatible with 16S-RMTase production in 120 clinical *Enterobacteriaceae* (Fig.1).

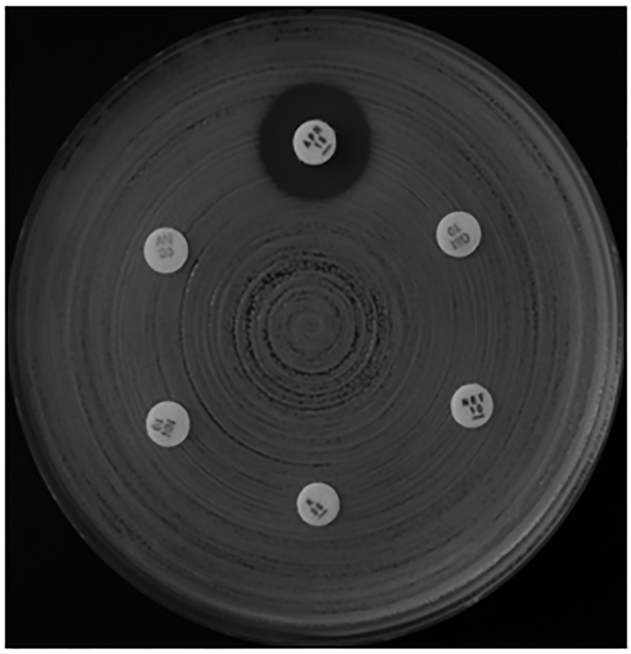

Figure 1: Typical image of 16S-RMTase-mediated resistance phenotype observed in clinical *Enterobacteriaceae* isolates at the cancer hospital. Clockwise: APR, apramycin; GM, gentamicin; NET, netilmicin; K, kanamycin; NN, tobramycin; AN, amikacin. The isolates exhibited concomitantly no inhibitory zone around all aminoglycosides tested, except apramycin.

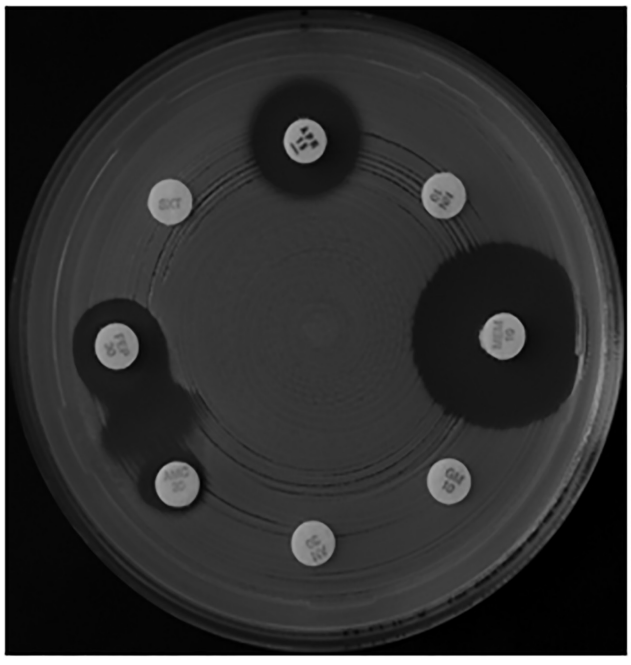

Figure 2: Antibiogram of *armA*-positive *Enterobacter cloacae* strain co-producing ESBL. Clockwise: APR, apramycin; NN, tobramycin; MEM, meropenem ; GM, gentamicin; AN, amikacin; AMC, amoxicillin/clavulanic acid; FEP, cefepime, SXT, trimethoprim/sulfamethoxazole.

All of them were confirmed to harbour 16S-RMTase-encoding genes in association with extended-spectrum β-lactamase (ESBL) genes as reported (3, 4, 5). In 16 of the 16S-RMTase-positive isolates co-production of metallo-β-lactamases (MBLs) was also determined as previously reported (3, 4, 5). Based on analysis of the observed resistance phenotypes and their corresponding genotypes we selected the following antibiotic disc indicators able to screen for 16S-RMTase and associated β-lactamase production that could be included in the routine antibiogram (Fig. 2). Both gentamicin and amikacin discs with no inhibitory zone, along with susceptibility to apramycin, proved indicative of 16S-RMTase production. Synergy between amoxicillin/clavulanic acid and cefepime proved indicative of ESBL production in all *Enterobacteriaceae*. Finally, meropenem disc was also included as an indicator of carbapenemase producion in accordance with the EUCAST guidelines for detection of resistance mechanisms (6).

DISCUSSION

16S rRNA methyltransferase-mediated aminoglycoside resistance, formerly confined to aminoglycoside producers as a mechanism of self-defence, has recently been identified in Gram-negative pathogens (1). In recent years, however, diverse 16S-RMTases have emerged as acquired resistance determinants in several genera of the *Enterobacteriaceae* and have been detected not only in humans, but also in animals (7). The genes encoding these determinants are usually associated with ESBLs but recently they have been found in association with carbapenemases resulting in difficult-to-treat multidrug-resistant pathogens (3, 4, 5, 7, 8). Thereby, it would be very important to continue monitoring the trend of 16S-RMTase producers under hospital and countrywide surveillance programs in both humans and animals to prevent their further global spread. In this connection, the development of practical screening techniques for detection of 16S-RMTase-producing *Enterobacteriaceae* will be of great assistance to the epidemiological study and rapid identification of such isolates in clinical microbiology laboratories.

In this study, we analysed resistance phenotypes of 120 well-characterised, consecutive 16S-RMTase-producing *Enterobacteriaceae*, collected at one hospital during an 11-years survey period (2004-2015) (3, 4, 5). Our results suggest that concomitant use of gentamicin, amikacin, and apramycin provide a reliable screening for 16S-RMTase production in all *Enterobacteriaceae*. Similar suggestion, as the two-step MIC

determination of both gentamicin and amikacin, was proposed from other researchers (7). Moreover, the addition of meropenem as an indicator of carbapenemase production and the double-disc synergy test for ESBL detection provided a good tool for further surveillance of 16S-RMTase-producing *Enterobacteriaceae* at the hospital level.

In conclusion, we have developed a cost-effective disc-based method to screen for 16S-RMTase producers and associated resistance markers within the routine antibiogram. Concomitant high level resistance to gentamicin and amikacin, but susceptibility to apramycin could be a sensitive screening tool for rapid detection, improved surveillance, and appropriate treatment of 16S-RMTase-producing multidrug-resistant enterobacterial pathogens.

ACKNOWLEDGEMENTS

We thank Mr. Stoyan Yordanov Atanasov for the precise photography of the antibiograms.

REFERENCES

1. Galimand M, Courvalin P, Lambert T. *Plasmid-mediated high-level resistance to aminoglycosides in Enterobacteriaceae due to 16S rRNA methylation*. Antimicrob Agents Chemother. 2003; 47:2565–2571.
2. Liou GF, Yoshizawa S, Courvalin P, Galimand M. *Aminoglycoside resistance by ArmA-mediated ribosomal 16S methylation in human bacterial pathogens*. J Mol Biol. 2006; 359:358-364.
3. Sabtcheva S, Saga T, Kantardjiev T, Ivanova M, Ishii Y, Kaku M. *Nosocomial spread of armA-mediated high-level aminoglycoside resistance in Enterobacteriaceae isolates producing CTX-M-3 β-lactamase in a cancer hospital in Bulgaria*. J Chemother. 2008; 20:593–599.
4. Ivanov I, Sabcheva S, Dobreva E, Todorova B, Velinov Tz, Borisova V, Petrova I, Ivancheva K, Asseva G, Padeshki P, Petrov P, Kantardjiev T. *Prevalence of carbapenemase genes among 16Sr RNA methyltransferase-producing Enterobacteriaceae isolated from cancer patients*. Probl. Inf. Parsit. Dis. 2014; 42(1):10-13.
5. Sabtcheva S, Ivanov I, Bozhana T, Ivanova K, Dobreva E, Kantardjiev T. *Prevalence of carbapenemase genes among 16S rRNA methyltransferase-producing Enterobacteriaceae isolated from cancer patients.* 26th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) 2016, April 9-12, Amsterdam, Netherlands, P-0716.
6. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Guideline for the detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance. Available from: http://www.eucast.org/resistance_mechanisms/.
7. Wachino J, Arakawa Y. *Exogenously acquired 16S rRNA methyltransferases found in aminoglycoside-resistant pathogenic Gram-negative bacteria: An update* Drug Resistance Updates 2012; 15:133-148.
8. Sabtcheva S, Kantardjiev T, Kaku M. *Characterization of conjugative plasmids mediating the dissemination of 16S ribosomal RNA methylases responsible for panaminoglycoside resistance of clinical Enterobacteriaceae in a Bulgarian cancer hospital.* Probl. Inf. Parasit. Dis. 2008; 36(2): 26-28.

COMPARISON OF TWO COMBINATION DISC TESTS FOR PHENOTYPIC DETECTION OF CARBAPENEMASE-PRODUCING *ENTEROBACTERIACEAE*

S. Sabtcheva[1], B. Todorova[1], I. N. Ivanov[2], K. Ivanova[2], V. Dobrinov[2], E. Dobreva[2], R. Hristova[2], M. Nedyalkov[2], T. Kantardjiev[2]

[1] Laboratory for Clinical Microbiology, Specialised hospital for active treatment in oncology, Sofia, Bulgaria
[2] National Reference Laboratory for Control and Monitoring of Antibiotic Resistance, Department of Microbiology National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria

ABSTRACT

Background: The prompt and accurate detection of carbapenemase-producing *Enterobacteriaceae* is essential for patient care and infection control procedures. Therefore, the introduction of a standardised method for routine carbapenemase detection in clinical diagnostic laboratories seems to be mandatory. The aim of the present study was to compare the performance of two commercially available combination disc tests for confirmation of carbapenemase production in *Enterobacteriaceae*, isolated in Bulgaria. **Material and methods**: Two commercial combination disc tests (CDTs): the MAST Carbapenemase Detection Set (MAST-CDS) and the KPC&MBL&OXA-48 disc kit were evaluated in comparison with molecular detection of carbapenemase genes. Test strains comprised 58 well-characterised *Enterobacteriaceae* from the collection of the National Reference Laboratory for Control and Monitoring of Antibiotic Resistance. Of these, 40 *Enterobacteriaceae* were confirmed to harbour various carbapenemase genes as follows: 17 bla_{KPC}-positive *Klebsiella pneumoniae*, 3 bla_{OXA-48}-positive *K. pneumoniae*, 11 bla_{NDM}-positive strains (4 *Escherichia coli* and 7 *K. pneumoniae*), and 9 bla_{VIM}-positive strains (6 *Proteus mirabilis*, 2 *Serratia marcescens,* and 1 *K. pneumoniae*). Furthermore, 18 carbapenem-non-susceptible and carbapenemase-non-producing *Enterobacteriaceae* were also included as negative controls. The tests were performed following the manufacturer's instructions. **Results**: Each of the two CDTs had 100% sensitivity for identification of class A (KPC) and class D (OXA-48) carbapenemases. In non-*Proteus* species, the sensitivity for class B carbapenemase detection was 100% when MAST-CDS was applied, while KPC&MBL&OXA-48 disc kit failed to detect the class B carbapenemase in VIM-positive *S.marcescens* strains. Both CDTs in this study failed to detect VIM production in *P. mirabilis* strains. The overall specificity was 100%. **Conclusions**: Our results indicate that the MAST-CDS combined with temocillin disc and the KPC&MBL&OXA-48 disc kit provide reliable phenotypic confirmation for class A, B, and OXA-48 carbapenemases in non-*Proteus Enterobacteriaceae* species isolated in Bulgaria. For phenotypic detection of VIM-producing *P. mirabilis* strains another method should be considered.

Keywords: *Enterobacteriaceae*, carbapenemase, phenotypic detection, combination disc test.

ADDRESS FOR CORRESPONDENCE:
Stefana Sabtcheva
Laboratory for Clinical Microbiology
Specialised Hospital for Active Treatment in Oncology
6 Plovdivsko pole Str
1756 Sofia, Bulgaria
Tel. 8076293
E-mail: stefanasabtcheva@gmail.com

INTRODUCTION

In *Enterobacteriaceae* resistance to carbapenems is mainly due to the production of carbapenemases of molecular class A, B, and D or, in rare cases, to the production of extended-spectrum β-lactamase (ESBL) and/or AmpC cephalosporinases combined with reduced permeability of the outer membrane due to loss or mutations in porins (1). Carbapenemases are β-lactamases that vary in their ability to hydrolyse all β-lactam antibiotics depending on the molecular class to which they belong (1, 2). The most common class A carbapenemases are the KPC-types initially confined to *Klebsiella pneumoniae* isolates, but afterwards continued to spread even outside the *Enterobacteriaceae* family. Carbapenemases of VIM-, IMP-, and NDM-types represent class B enzymes. Besides *Enterobacteriaceae*, they are frequently isolated from multidrug-resistant *Pseudomonas* spp. and *Acinetobacter* spp. as well. Class D enzymes are mainly represented by OXA-48-like producers identified mainly in *Enterobacteriaceae*. Genes encoding carbapenemases are often associated with mobile genetic elements, located on plasmids

which also carry fluoroquinolone, aminoglycoside, and other resistance determinants (2). This facilitates their rapid dissemination and seriously compromises the treatment of life-threatening infections which is often limited to colistin and tigecycline (3). Therefore, rapid detection of carbapenemase-producers becomes essential for infection control purposes, successful treatment of patients and the preservation of carbapenem efficacy (1). Although the molecular identification of carbapenemase genes remains the "gold standard", phenotypic assessment using the routine antibiogram provided reliable and cost effective method for rapid detection of carbapenemase production (4). In Bulgaria, commercially available combination disc tests were recently introduced for phenotypic detection and differentiation of carbapenemases, based on specific inhibition of the hydrolytic activity of class A and B enzymes. As yet there is no specific inhibitor of class D enzymes, their differentiation is based on the established high level resistance to temocillin defined as phenotypic indicator of production of OXA-48-like carbapenemases (5).

The aim of the present study was to compare the performance of two commercially available combination disk tests for confirmation of carbapenemase production in *Enterobacteriaceae*, isolated in Bulgaria.

MATERIALS AND METHODS

The study was performed with 58 clinical *Enterobacteriaceae* isolates*,* with inhibition zone diameter around 10 µg meropenem disc <25 mm (4), subdivided in two groups: carbapenemase-positive and carbapenemase-negative. The first group included 40 strains with already confirmed genotypes by Real-time PCR and sequencing as follows: 17 bla_{KPC}-positive *Klebsiella pneumoniae*, 3 bla_{OXA-48}-positive *K. pneumoniae*, 11 bla_{NDM}-positive strains (4 *Escherichia coli* and 7 *K. pneumoniae*), and 9 bla_{VIM}-positive strains (6 *Proteus mirabilis*, 2 *Serratia marcescens,* and 1 *K. pneumoniae*). Furthermore, 18 carbapenem-non-susceptible and carbapenemase-non-producing *Enterobacteriaceae* were also included as negative controls. They were confirmed biochemically by the Carba NP test (6).

Phenotypic detection and confirmation of carbapenemase production was performed in parallel with the MASTDISCS™ ID carbapenemase (*Enterobacteriaceae*) detection disc set (MAST Diagnostics, Merseyside, UK) and the KPC&MBL&OXA-48 disc kit (Liofilchem, Roseto degli Abruzzi, Italy), following the manufacturer's instructions. Briefly, Mueller-Hinton II agar plate was inoculated with overnight culture suspension of the test organism equal to 0.5 McFarland standard. Disc meropenem 10 µg, disc meropenem 10 µg with phenylboronic acid as a KPC inhibitor, disc meropenem 10 µg with cloxacillin as an AmpC inhibitor, disc meropenem 10 µg with dipicolinic acid as a class B metallo-β-lactamase (MBL) inhibitor and disc temocillin 30 µg were placed on inoculated agar plate. Following incubation at 36°C for 18–24 hours, the differences between inhibition zones around the meropenem disc compared to the meropenem discs combined with inhibitors were determined in mm. The test was considered positive for KPC production when the diameter of the growth-inhibitory zone around the meropenem+phenylboronic acid disc was ≥4 mm larger than that around the meropenem disc alone. The test was considered positive for MBL production when the diameter of the growth-inhibitory zone around the meropenem+dipicolinic acid disc was ≥5 mm larger than that around the meropenem disc alone. Finally, the concomitant absence of synergy with any inhibitor and zone diameter <11 mm around the temocillin disc was interpreted as suspicious for OXA-48-like production. As MASTDISCS™ ID carbapenemase detection disc set does not include temocillin disc, it was additionally supplied by MAST in order to obtain comparable results.

RESULTS

Performance results for the two combination disk tests - the MAST Carbapenemase Detection Set (MAST-CDS) and the KPC&MBL&OXA-48 disc kit were easy to interpret and correctly classified all KPC producers to Ambler class A of enzymes, showing significant increase of the growth-inhibitory zone around the meropenem+phenylboronic acid discs compared to that around the meropenem-alone disc (Fig.1).

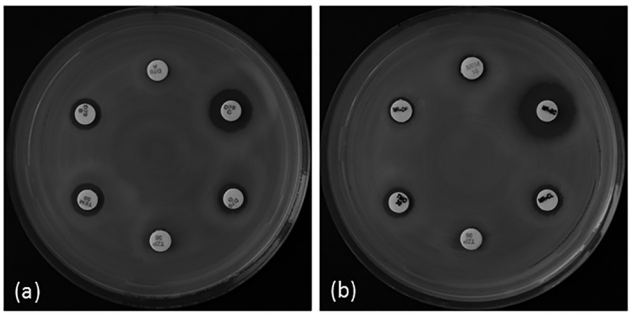

Figure 1: Positive combination disc test for a KPC-producing *K. pneumoniae* isolate (a) using MAST-CDS; (b) using KPC&MBL&OXA-48 disc kit. Clockwise: A/MEM, meropenem; C/MR+BO, meropenem with phenylboronic acid; D/MR+CL, meropenem with cloxacillin; TZP, piperacillin/tazobactam; TEM/TMO, temocillin; B/MR+DP, meropenem with dipicolinic acid.

Regarding MBL producers some discrepancy was observed between the two combination disk tests. MAST-CDS correctly classified all NDM- and VIM-producing non-*Proteus* species to class B enzymes, whereas the KPC&MBL&OXA-48 disc kit was not able to confirm VIM-production in *S.marcescens* strains (Fig.2 and Fig.3). The both CDTs studied failed to detect VIM production in *P. mirabilis* strains (Fig.4).

Finally, the both CDTs correctly classified all OXA-48 producers to class D of enzymes based on the concomitant absence of synergy with any inhibitors and zone diameter 6 mm with temocillin disc (Fig5). At the same time, no synergy with any of the inhibitors was observed among the18 carbapenemase non-producers rending an overall specificity of 100% for the two combination disk tests studied.

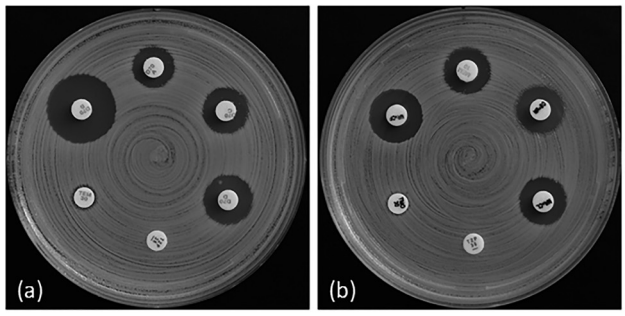

Figure 2: Positive combination disc test for a NDM-producing *K. pneumoniae* isolate (a) using MAST-CDS; (b) using KPC&MBL&OXA-48 disc kit. Clockwise: A/MEM, meropenem; C/MR+BO, meropenem with phenylboronic acid; D/MR+CL, meropenem with cloxacillin; TZP, piperacillin/tazobactam; TEM/TMO, temocillin; B/MR+DP, meropenem with dipicolinic acid.

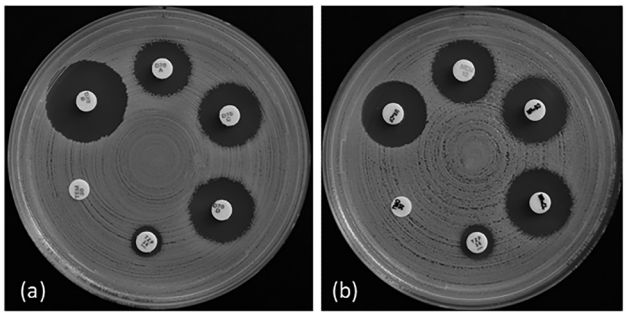

Figure 3: (a) Positive combination disc test for a VIM-producing *S. marcescens* isolate using MAST-CDS; (b) Negative combination disc test for a VIM-producing *S. marcescens* isolate using KPC&MBL&OXA-48 disc kit. Clockwise: A/MEM, meropenem; C/MR+BO, meropenem with phenylboronic acid; D/MR+CL, meropenem with cloxacillin; TZP, piperacillin/tazobactam; TEM/TMO, temocillin; B/MR+DP, meropenem with dipicolinic acid.

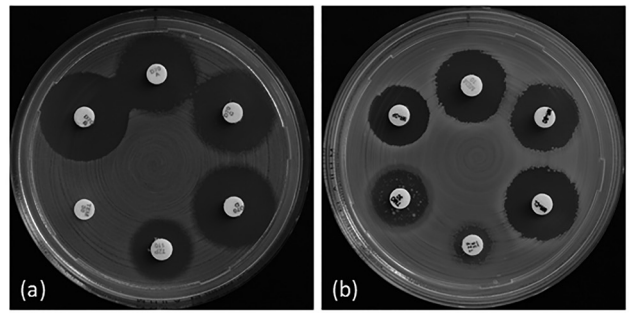

Figure 4: Negative combination disc test for a VIM-producing *P. mirabilis* isolate (a) using MAST-CDS; (b) using KPC&MBL&OXA-48 disc kit. Clockwise: A/MEM, meropenem; C/MR+BO, meropenem with phenylboronic acid; D/MR+CL, meropenem with cloxacillin; TZP, piperacillin/tazobactam; TEM/TMO, temocillin; B/MR+DP, meropenem with dipicolinic acid.

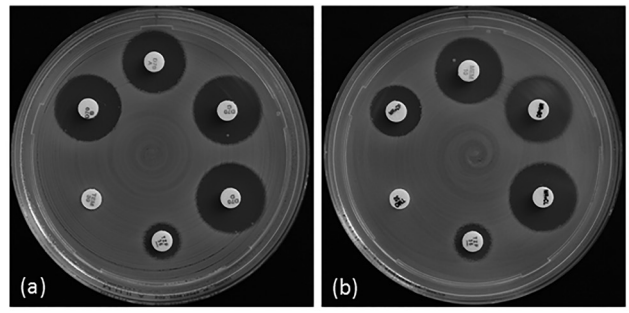

Figure 5: Positive combination disc test for a OXA-48-producing *K. pneumoniae* isolate (a) using MAST-CDS; (b) using KPC&MBL&OXA-48 disc kit. Clockwise: A/MEM, meropenem; C/MR+BO, meropenem with phenylboronic acid; D/MR+CL, meropenem with cloxacillin; TZP, piperacillin/tazobactam; TEM/TMO, temocillin; B/MR+DP, meropenem with dipicolinic acid.

DISCUSSION

Rapid and accurate detection of carbapenemase-producing *Enterobacteriaceae* is essential for patient treatment and infection control management. This necessitates the introduction of a standardised, reliable, and cost effective method for routine detection of carbapenemases in clinical microbiology laboratories. Unfortunately the process is seriously hampered by the low-level resistance to carbapenems, conferred by the corresponding genes, which also facilitates their rapid dissemination. The efforts of many researchers aim at developing efficient algorithms for phenotypic detection and differentiation of carbapenemase-producers.

In our previous study, following the characterisation of the first OXA-48-positive *K. pneumoniae* strain in Bulgaria, we developed an algorithm for routine identification of the difficult-to-detect

OXA-48-positive/ESBL-negative phenotype (7, 8). In another study we evaluated the effectiveness of the KPC&MBL&OXA-48 disc kit (Liofilchem, Italy) for detection and differentiation of KPC-producing clinical *K. pneumoniae* strains, isolated in the country. We found that this test, like other commercial standardised tests, identifies KPC-producers with 100% sensitivity and specificity (9).

The current study focused on the comparative performance of two commercially available combination disc tests for confirmation of carbapenemase production in *Enterobacteriaceae* isolated in Bulgaria. In line with studies from other authors (4), KPC-producing *K. pneumoniae*, OXA-48-producing *K. pneumoniae,* and NDM-producing *Enterobacteriaceae* were correctly confirmed with 100% sensitivity and specificity. Unfortunately, our results support another trend concerning the difficulty in detecting IMP- and VIM-producers as a whole and particularly in *P. mirabilis* strains (2, 10). The reason for this phenomenon is again indicative of the low-level resistance to carbapenems.

In conclusion, our results indicate that the MAST-CDS combined with temocillin disc and the KPC&MBL&OXA-48 disc kit provide reliable phenotypic confirmation for class A, B, and OXA-48 carbapenemases in non-*Proteus Enterobacteriaceae* species isolated in Bulgaria. For phenotypic detection of VIM-producing *P. mirabilis* strains another method should be considered.

ACKNOWLEDGEMENTS

We thank Mr. Stoyan Yordanov Atanasov for the precise photography of the antibiograms.

REFERENCES

1. Levy Hara G, Gould I, Endimiani A, Pardo PR, Daikos G, Hsueh PR, et al. *Detection, treatment, and prevention of carbapenemase-producing Enterobacteriaceae*: Recommendations from an International Working Group. J Chemother. 2013; 25(3):129-140.
2. Doyle D, Peirano G, Lascols C et al. *Laboratory detection of Enterobacteriaceae that produce carbapenemases.* J Clin Microbiol 2012; 50:3877-3880.
3. Livermore DM, Warner M, Mushtaq S et al. *What remains against carbapenem-resistant Enterobacteriaceae? Evaluation of chloramphenicol, ciprofloxacin, colistin, fosfomycin, minocycline, nitrofurantoin, temocillin and tigecycline*. Int J Antimicrob Agents 2011; 37:415-419.
4. EUCAST Guidelines for Detection of Resistance Mechanisms and Specific Resistances of Clinical and/or Epidemiological Importance Version 1.0. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Resistance_mechanisms/EUCAST_detection_of_resistance_mechanisms_v1.0_20131211
5. Huang T-D, Poirel L, Bogaerts P, Berhin C, Nordmann P, Glupczynski Y. *Temocillin and piperacillin/tazobactam resistance by disc diffusion as antimicrobial surrogate markers for the detection of carbapenemase-producing Enterobacteriaceae in geographical areas with a high prevalence of OXA-48 producers*. J Antimicrob Chemother. 2014; 69(2):445-450.
6. Ivanova K, I. N. Ivanov, S. Sabtcheva et al. *Evaluation of the Carba NP test for detection of carbapenemase-producing Enterobacteriaceae: Preliminary results*. Probl Inf Parsit Dis 2015; 43(2):12-14.
7. Sabtcheva S, Ivanov IN, B.Todorova et al. *Detection and characterization of OXA-48-producing Klebsiella pneumoniae originated in Bulgaria.* J Chemother 2015 May 27:DOI 10.1179/1973947815Y.0000000047.
8. Sabtcheva S. *Phenotypic detection of OXA-48-producing enterobacterial isolates by routine antibiogram.* Probl Inf Parasit Dis 2015; 43(1):5-7.
9. Sabtcheva S. et al. *Routine laboratory detection of Klebsiella pneumoniae carbapenemase-producing Enterobacteriaceae.* Probl Inf Parsit Dis 2015; 43(2):5-7.
10. Saito R et al. *Evaluation of a simple phenotypic method for the detection of carbapenemase-producing Enterobacteriaceae*. J Microbiol Methods 2015; 108 ;45-48.

PHENOTYPIC DETECTION OF AAC(6')-IB-CR-PRODUCING *ENTEROBACTERIACEAE* BY ROUTINE ANTIBIOGRAM

S. Sabtcheva

Laboratory for Clinical Microbiology, Specialised hospital for active treatment in oncology, Sofia, Bulgaria

ABSTRACT

Plasmid-mediated quinolone resistance (PMQR) in *Enterobacteriaceae* has increased in the last years, seriously compromising the management of life-threatening infections. PMQR genes (*qnr*, *aac(6 ,)-Ib-cr*, *qepA* and *oqxAB*) confer low-level quinolone resistance and are frequently co-transmitted with extended-spectrum β-lactamase genes. AAC(6)-Ib-cr-mediated aminoglycoside and fluoroquinolone resistance was first detected in 2006, but is now recognised to be widely disseminated. In the present study, we attempted to develop a disc-based method to screen for AAC(6)-Ib-cr producers, based on the peculiar substrate profile of the AAC(6')-Ib-cr enzyme. We analysed resistance phenotypes of 52 well-characterised *aac(6 ,)-Ib-cr*-positive *Enterobacteriaceae*, collected at a cancer hospital. Our results show that resistance to tobramycin along with reduced susceptibility to amikacin and ciprofloxacin, but susceptibility to gentamicin, nalidixic acid and levofloxacin provide a reliable screening for AAC(6')-Ib-cr producion in *Enterobacteriaceae*. The proposed antibiotic disc configuration reflects the substrate profile of AAC(6')-Ib-cr enzyme and could be useful for early detection of AAC(6)-Ib-cr-positive/ quinolone mutation-negative isolates. We conclude that the developed disc-based method is a simple cost-effective screening tool for rapid detection and appropriate treatment of AAC(6')-Ib-cr-producing multidrug-resistant enterobacterial pathogens.

ADDRESS FOR CORRESPONDENCE:
Stefana Sabtcheva
Laboratory for Clinical Microbiology
Specialised Hospital for Active Treatment in Oncology
6 Plovdivsko pole Str
1756 Sofia, Bulgaria
Tel. 8076293
E-mail: stefanasabtcheva@gmail.com

Keywords: *Enterobacteriaceae*, acquired quinolone resistance, AAC(6')-Ib-cr

INTRODUCTION

Plasmid-mediated quinolone resistance (PMQR) is an emerging healthcare concern, especially in *Enterobacteriaceae*, involving mechanisms of target protection (*qnr*), antbiotic inactivation (*aac(6 ,)-Ib-cr*) or active efflux systems (*qepA* and *oqxAB*) (1). The PMQR determinants confer only low-level quinolone resistance, but the *aac(6')-Ib-cr* carriage could facilitate the selection of higher level chromosomal resistance mutations when quinolones are used (2). To date, no specific phenotypic tests for detection of PMQR mechanisms are available.

In the present study, we attempted to develop a disc-based method to screen for
AAC(6)-Ib-cr producers, based on the peculiar substrate profile of the AAC(6')-Ib-cr enzyme.

MATERIALS AND METHODS

A total of 52 isolates of *Escherichia coli* (n=47), *Citrobacter freundii* (n=2), *Klebsiella pneumoniae* (n=1), *Enterobacter aerogenes* (n=1) and *Morganella morganii* (n=1), already confirmed to harbour the *aac(6')-Ib-cr* gene by PCR and sequencing were studied (3, 4). The isolates were tested using disc diffusion according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (5) with ciprofloxacin 5 μg, norfloxacin 10 μg, levofloxacin 5 μg, nalidixic acid 30 μg, amikacin 30 μg, tobramycin 10 μg, netilmicin 10 μg, kanamycin 30 μg and gentamicin 10 μg discs (Becton Dickinson, Sparks, MD). The results were interpreted according to EUCAST breakpoints (6), except for nalidixic acid and kanamycin zone diameters that were interpreted using Clinical and Laboratory Standards Institute (CLSI) breakpoints (7).
E. coli ATCC 25922 was used as antibiotic-susceptible control.

RESULTS AND DISCUSSION

The common aminoglycoside acetyltransferase gene *aac(6')-Ib* confers resistance to kanamycin, tobramycin, netilmicin and amikacin but not to gentamicin (2). According to EUCAST expert rules v. 2.0, rule no. 12.7., production of acquired AAC(6')-I-like enzyme confers phenotypic resistance to tobramycin, kanamycin, netilmicin, but may not confer phenotypic amikacin resistance despite modification of amikacin (8). Indeed, our susceptibility testing results showed that all isolates were resistant to tobramycin, kanamycin, netilmicin,

but 75% (39/52) of the isolates exhibited only reduced susceptibility to amikacin as shown in Figure 1. In addition, the *cr* variant of the common acetyltransferase gene - *aac(6')-Ib-cr* confers low-level resistance to ciprofloxacin and norfloxacin but not to nalidixic acid and the other fluoroquinolones like levofloxacin, as indicated in Figure 2.

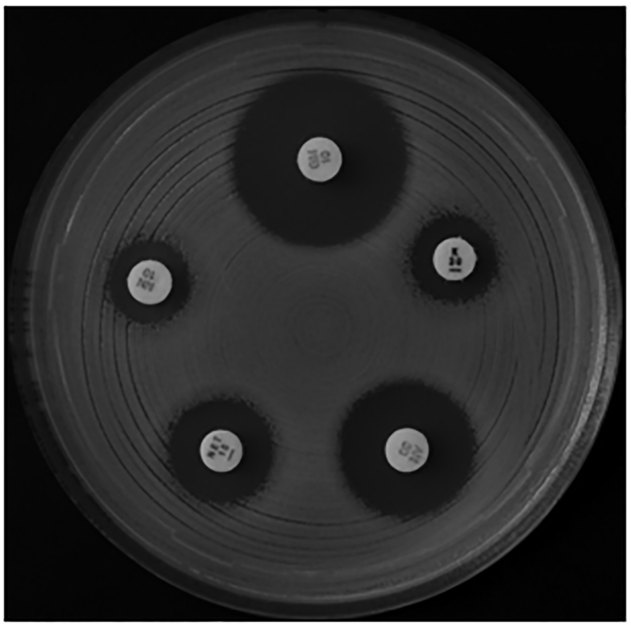

Figure 1: Typical image of aminoglycoside resistance phenotype due to AAC(6')-Ib production observed in clinical *Enterobacteriaceae* isolated at the cancer hospital. Clockwise: GM, gentamicin; K, kanamycin; AN, amikacin; NET, netilmicin; NN, tobramycin. The isolates exhibited resistance to tobramycin, kanamycin and netilmicin, reduced susceptibility to amikacin, and were susceptible to gentamicin.

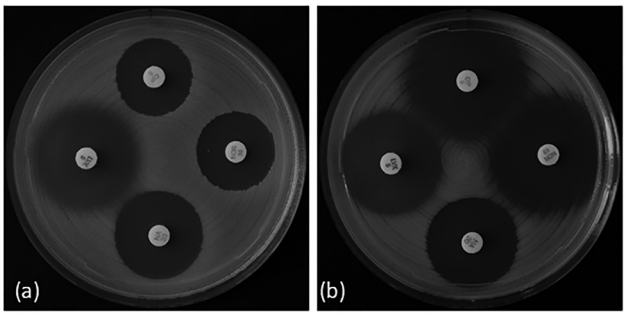

Figure 2: (a) Typical image of resistance phenotype due to AAC(6')-Ib-cr enzyme concerning quinolones. Clockwise: CIP, ciprofloxacin; NOR, norfloxacin; NA, nalidixic acid; LVX, levofloxacin. The isolate exhibited reduced susceptibility to both ciprofloxacin and norfloxacin, but remained susceptible to nalidixic acid and levofloxacin. (b) Typical image of quinolone "susceptible" resistance phenotype, shown for comparison.

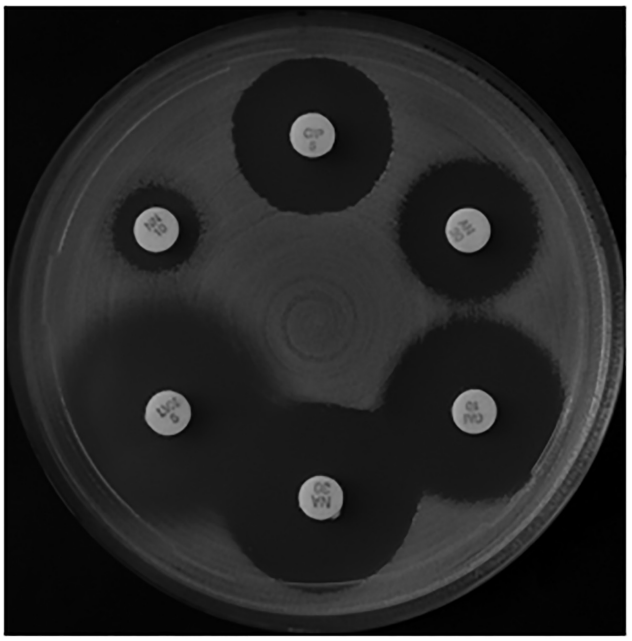

Figure 3: Antibiogram of *aac(6')Ib-cr*-positive *E. aerogenes* strain. Clockwise: CIP, ciprofloxacin; AN, amikacin; GM, gentamicin; NA, nalidixic acid; LVX, levofloxacin;
NOR, norfloxacin; NN, tobramycin. Resistance to tobramycin along with reduced susceptibility to amikacin and ciprofloxacin, but susceptibility to gentamicin, nalidixic acid and levofloxacin are indicative of AAC(6')-Ib-cr production.

Based on analysis of the observed resistance phenotypes and their corresponding genotypes we selected tobramycin, amikacin, ciprofloxacin as antibiotic disc indicators along with gentamicin, nalidixic acid, levofloxacin as anti-indicators able to screen for AAC(6')-Ib-cr producion (Fig.3) Our results show that resistance to tobramycin along with reduced susceptibility to amikacin and ciprofloxacin, but susceptibility to gentamicin, nalidixic acid and levofloxacin provide a reliable screening for AAC(6')-Ib-cr producion in *Enterobacteriaceae*. The proposed antibiotic disc configuration reflects the substrate profile of the AAC(6')-Ib-cr enzyme and could be useful for early detection of AAC(6)-Ib-cr-positive/quinolone mutation-negative isolates.
We conclude that the developed disc-based method is a simple cost-effective screening tool for rapid detection and appropriate treatment of AAC(6')-Ib-cr-producing multidrug-resistant enterobacterial pathogens.

ACKNOWLEDGEMENTS
We thank Mr. Stoyan Yordanov Atanasov for the precise photography of the antibiograms.

REFERENCES

1. Strahilevitz J, Jacoby GA, Hooper DC, Robicsek A. *Plasmid-Mediated Quinolone Resistance: a Multifaceted Threat*. Clin Microbiol Rev. 2009; 22(4):664–689.
2. Robicsek A, Strahilevitz J, Jacoby GA, Macielag M, Abbanat D, Bush K, Hooper DC. *Fluoroquinolone modifying enzyme: a novel adaptation of a common aminoglycoside acetyltransferase*. Nat. Med. 2006; 12:83-88.
3. Sabtcheva S, Kaku M, Saga T, Ishii Y, Kantardjiev T. *High prevalence of the aac(6')-Ib-cr gene and its dissemination among Enterobacteriaceae by CTX-M-15 plasmids in Bulgaria*. Antimicrob. Agents Chemother. 2009; 53(1):335-336.
4. Sabtcheva S, Kantardjiev T, Ivanova M, Kaku M. *Prevalence of the fluoroquinolone-modifying acetyltransferase gene aac(6')-Ib-cr among extended-spectrum beta-lactamase-producing enterobacterial isolates in a cancer hospital in Bulgaria*. Probl Infect Parasit Dis. 2008; 36(1):21-22.
5. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Disk Diffusion Test Methodology. Available from: http://www.eucast.org/ast_of_bacteria/disk_diffusion_methodology/ .
6. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Breakpoint tables for interpretation of MICs and zone diameters. Available from: http://www.eucast.org/clinical-breakpoints/.
7. CLSI. Performance standards for antimicrobial susceptibility testing; Twentyfifth informational supplement. CLSI document M100-S25. Wayne, PA: Clinical and Laboratory Standards Institute; 2015.
8. Leclercq R, Cantón R, Brown DF, Giske CG, Heisig P, MacGowan AP, Mouton JW, Nordmann P, Rodloff AC, Rossolini GM, Soussy CJ, Steinbakk M, Winstanley TG, Kahlmeter G. *EUCAST expert rules in antimicrobial susceptibility testing*. Clin Microbiol Infect. 2013;19(2):141-60.

COMPARISON OF EUROPEAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING AND CLINICAL LABORATORY STANDARDS INSTITUTE CRITERIA FOR THE INTERPRETATION OF EXTENDED-SPECTRUM B-LACTAMASE-PRODUCING *ENTEROBACTERIACEAE* ISOLATED AT A CANCER HOSPITAL

S. Sabtcheva[1], B. Todorova[1], T. Kantardjiev[2]

[1] Laboratory for Clinical Microbiology, Specialised hospital for active treatment in oncology, Sofia, Bulgaria
[2] National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria

ABSTRACT

Background: In 2010 European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) eliminated the need for routine extended-spectrum β-lactamase (ESBL) detection and reporting β-lactam susceptibility testing results accordingly. The aim of this study was to determine how many ESBL-producing *Enterobacteriaceae* test susceptible using EUCAST and CLSI clinical breakpoints. **Material and methods**: One hundred consecutive *Enterobacteriaceae* with ESBL phenotype were collected at the Specialised hospital for active treatment in oncology between August 2015 and June 2016. ESBL production was systematically determined on the basis of observation of a synergy zone between amoxicillin/clavulanic acid and cefepime and/or ceftazidime and/or cefotaxime as described in the EUCAST guidelines for detection of resistance mechanisms. The collection comprised 40 *Escherichia coli*, 25 *Klebsiella pneumoniae*, 12 *Enterobacter cloacae*, 12 *Serratia marcescens*, 7 *Klebsiella oxytoca*, 2 *Citrobacter freundii,* and single isolates of *Enterobacter aerogenes* and *Morganella morganii*. Disc diffusion antimicrobial susceptibility testing was performed and interpreted according to EUCAST 2015 and CLSI 2015 guidelines. **Results**: All 100 ESBL-producing isolates were non-susceptible to ceftriaxone and cefotaxime if EUCAST and CLSI zone diameter clinical breakpoints were applied, whereas 6% of all (5/40 *E. coli* and 1/25 *K. pneumoniae*) were susceptible to cefepime according to both breakpoints. However, significant differences in the susceptibility rates of ceftazidime and aztreonam were demonstrated applying both guidelines. According to EUCAST 2015 breakpoints 4% of all ESBL-producers (3/40 *E. coli* and 1/25 *K. pneumoniae*) were susceptible to ceftazidime and aztreonam, whereas, according to CLSI 2015 clinical breakpoints 22% of all (18/40 *E. coli*, 2/12 *S. marcescens*, 1/25 *K. pneumoniae*, 1/1 *M. morganii*) were susceptible to ceftazidime and 19% (17/40 *E. coli*, 1/25 *K. pneumoniae*, 1/1 *M. morganii*) were susceptible to aztreonam. **Conclusions**: All ESBL-producing *Enterobacteriaceae* isolated at the cancer hospital would be reported to be non-susceptible to cefotaxime and ceftriaxone applying the EUCAST and CLSI 2015 clinical breakpoints, but a substantial number of ESBL-producing *E. coli* strains would be reported to be susceptible to ceftazidime and aztreonam, according to CLSI 2015 clinical breakpoints. Further harmonisation is needed to cease the controversial reporting of ceftazidime and aztreonam in therapy recommendations when using the EUCAST and CLSI guidelines.

Keywords**:** *Enterobacteriaceae*, extended-spectrum β-lactamase, interpretive criteria

ADDRESS FOR CORRESPONDENCE:
Stefana Sabtcheva
Laboratory for Clinical Microbiology
Specialised Hospital for Active Treatment in Oncology
6 Plovdivsko pole Str
1756 Sofia, Bulgaria
Tel. 8076293
E-mail: stefanasabtcheva@gmail.com

INTRODUCTION

Enterobacteriaceae, especially those producing extended-spectrum β-lactamases (ESBLs), can cause various nosocomially acquired life-threatening infections (1). Until 2009, antimicrobial susceptibility testing (AST) guidelines recommended routine ESBL detection and reporting *in vitro* susceptible and intermediate AST results for penicillins, cephalosporins, and monobactams in ESBL-producing isolates as resistant (2, 3). In 2010 European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical

and Laboratory Standards Institute (CLSI) eliminated the need for routine ESBL testing and allowed the treatment of ESBL-producing isolates with cephalosporins which tested susceptible according to the revised versions of the EUCAST and CLSI guidelines.

The aim of this study was to determine how many recent ESBL-producing clinical *Enterobacteriaceae* isolates test susceptible using the current EUCAST and CLSI clinical breakpoints.

MATERIALS AND METHODS

The study was carried out at the Specialised hospital for active treatment in oncology, Laboratory for Clinical Microbiology between August 2015 and June 2016. During this period, 100 consecutive clinical *Enterobacteriaceae* isolates with ESBL phenotype were collected. The ESBL production was routinely determined on the basis of observation of a synergy zone between amoxicillin/clavulanic acid and cefepime and/or ceftazidime and/or cefotaxime as described in the EUCAST guidelines for detection of resistance mechanisms (4). Identification to the species level was determined by Vitek-2 (bioMérieux, Marcy l'Etoile, France). Disc diffusion antimicrobial susceptibility testing was performed on Mueller-Hinton II agar plates (Becton Dickinson, Sparks, MD, USA) using overnight cultures with a turbidity equivalent to 0.5 McFarland standard followed by incubation at 35°C for 18h. Antibiotic discs supplied by Becton Dickinson were used. Results were interpreted according to the EUCAST 2015 and CLSI 2015 guidelines (5, 6).

RESULTS

The 100 ESBL-producing isolates were recovered from the following specimens: wound (n=56), urine (n=34), respiratory fluid (n=6), and peritoneal fluid (n=4). ESBLs were found in eight different species. The major ESBL-producers were *Escherichia coli* (40%) and *Klebsiella pneumoniae* (25%), followed by *Enterobacter cloacae* (12%), *Serratia marcescens* (12%), *Klebsiella oxytoca* (7%), *Citrobacter freundii* (2%), and single isolates of *Enterobacter aerogenes* and *Morganella morganii*. In Table 1 are summarised the comparative inhibition zone interpretation results of cephalosporins and aztreonam for 100 ESBL-producers according to EUCAST 2015 and CLSI 2015 breakpoints. Equal patterns of susceptibility to ceftriaxone and cefotaxime were detected applying the EUCAST 2015 and CLSI 2015 guidelines. None of the ESBL-producers were interpreted as "susceptible". Equal rates of susceptibility (6% of all) were demonstrated for cefepime applying both guidelines. Cefepime "susceptible" isolates comprised 5/40 *E. coli* and 1/25 *K. pneumoniae,* but none of the chromosomal AmpC *Enterobacteriaceae*. However, ESBL-producing isolates had significantly different susceptibility rates to ceftazidime and aztreonam when comparing EUCAST 2015 with CLSI 2015 breakpoints (4% versus 22% ceftazidime "susceptible", and 4% versus 19% aztreonam "susceptible", respectively). In detail, 3/40 *E. coli* and 1/25 *K. pneumoniae* tested susceptible for ceftazidime and aztreonam according to EUCAST interpretive criteria, whereas 18/40 *E. coli*, 2/12 *S. marcescens*, 1/25 *K. pneumoniae*, and 1/1 *M. morganii* were susceptible to ceftazidime and 17/40 *E. coli*, 1/25 *K. pneumoniae*, 1/1 *M. morganii* were susceptible to aztreonam according to CLSI 2015 clinical breakpoints. Of note, the discrepancy between EUCAST and CLSI susceptibility testing results were observed mainly among ESBL-producing *E. coli* isolates.

DISCUSSION

In 2010 EUCAST and CLSI eliminated the need for routine ESBL detection and reporting β-lactam susceptibility testing results accordingly. The treatment of ESBL-producing isolates with cephalosporins and aztreonam is allowed depending on the individual susceptibility testing categorisation. However, few data are available showing antibiotic susceptibility patterns for defined populations of ESBL-producing isolates according to revised EUCAST and CLSI guidelines (7, 8).

This study describes various resistance patterns for ESBL phenotype/species combinations if EUCAST 2015 and CLSI 2015 zone diameter breakpoints are applied (Table 1). Lack of susceptibility to cefotaxime and ceftriaxone was always found for all ESBL-producing isolates, whereas ceftazidime was categorised as susceptible in 4% and 22%, respectively, if EUCAST 2015 and CLSI 2015 breakpoints were applied. Similarly, aztreonam was categorised as susceptible in 4% and 19%, respectively, according to EUCAST 2015 and CLSI 2015 breakpoints, whereas cefepime was categorised as susceptible only in 6% of ESBL-producers if both 2015 guidelines were applied. Our results are in concordance with other studies and reflect the current worldwide dominance of CTX-M-type enzymes (8, 9, 10). The variation in susceptibility patterns of cef-

Table 1. Comparison of antibiotic susceptibility profiles of 100 ESBL-producing clinical *Enterobacteriaceae* isolates according to the EUCAST 2015 and CLSI 2015 breakpoints.

Drug/ interpretation (%)		EUCAST 2015			CLSI 2015		
		E.coli n=40	*Klebsiella* spp. n=32	Chromosomal AmpC *Enterobacteriaceae*[a] n=28	*E.coli* n=40	*Klebsiella* spp. n=32	Chromosomal AmpC *Enterobacteriaceae*[a] n=28
Ceftriaxone	R	38	32	28	38	32	28
	I	2	0	0	2	0	0
	S	0	0	0	0	0	0
Cefotaxime[b]	R	38	32	28	38	32	28
	I	2	0	0	2	0	0
	S	0	0	0	0	0	0
Ceftazidime[b]	R	32	31	25	20	26	25
	I	5	0	3	2	5	0
	S	3	1	0	18	1	3
Cefepime	R	25	31	26	24	31	24
	I	10	0	2	11	0	4
	S	5	1	0	5	1	0
Aztreonam	R	23	31	27	22	31	25
	I	14	0	1	1	0	2
	S	3	1	0	17	1	1

R, resistant; I, intermediate; S, susceptible

[a] Isolates include: 12 *E. cloacae*, 12 *S. marcescens*, 2 *C. freundii*, 1 *E. aerogenes,* and 1 *M. Morganii.*

[b] For these drugs, EUCAST and CLSI use different disc contents: cefotaxime (EUCAST 5 µg/disc, CLSI 30 µg/disc), ceftazidime (EUCAST 10 µg/disc, CLSI 30 µg/disc). Isolates were tested with both disc contents in parallel and interpretation was carried out accordingly.

tazidime could be attributed to the lower antibiotic disc content recommended by EUCAST while retaining almost the same breakpoint values as those in CLSI. Differences in aztreonam categorisation presumably arise due to setting the resistance breakpoint in EUCAST at a value corresponding to the susceptibility breakpoint in CLSI.

A limitation of this study was the local origin of the clinical strains and the absence of ESBL genotype determination. However, even suggested from the phenotype, our results underline that different ESBL/species combinations produce distinct antibiotic susceptibility patterns. Thus, further studies are needed to characterise the relationship between ESBL production, antimicrobial susceptibility and clinical outcome.

In conclusion, all ESBL-producing *Enterobacteriaceae* isolated at the cancer hospital would be reported to be non-susceptible to cefotaxime and ceftriaxone applying the EUCAST and CLSI 2015 clinical breakpoints, but a substantial number of ESBL-producing *E. coli* strains would be reported to be susceptible to ceftazidime and aztreonam, according to CLSI 2015 clinical breakpoints. Further harmonisation is needed to cease the controversial reporting of ceftazidime and aztreonam in therapy recommendations when using the EUCAST and CLSI guidelines.

REFERENCES

1. Pitout JD. *Infections with extended-spectrum β-lactamase-producing Enterobacteriaceae: changing epidemiology and drug treatment choices*. Drugs 2010; 70: 313-33.
2. Clinical Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing: Nineteenth Informational Supplement M100-S19. CLSI, Wayne, PA, USA, 2009.
3. EUCAST. Expert Rules in Antimicrobial Susceptibility Testing. Version 1. 2008. http://www.eucast.org/expert_rules.
4. European Committee on Antimicrobial Susceptibility Testing

(EUCAST). Guideline for the detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance. Available from: http://www.eucast.org/resistance_mechanisms/.
5. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Breakpoint tables for interpretation of MICs and zone diameters. Available from: http://www.eucast.org/clinical-breakpoints/.
6. CLSI. Performance standards for antimicrobial susceptibility testing; Twentyfifth informational supplement. CLSI document M100-S25. Wayne, PA: Clinical and Laboratory Standards Institute; 2015.
7. Wang P, Hu F, Xiong Z et al. *Susceptibility of extended-spectrum β-lactamase-producing Enterobacteriaceae according to the new CLSI breakpoints*. J Clin Microbiol 2011; 49:3127-3131.
8. Hombach M, Mouttet B, Bloemberg GV. *Consequences of revised CLSI and EUCAST guidelines for antibiotic susceptibility patterns of ESBL- and AmpC β-lactamase-producing clinical Enterobacteriaceae isolates*. J Antimicrob Chemother 2013; 68:2092-2098.
9. Markovska R, Keuleyan E, Ivanova D, Markova B, Proevska J, Leseva M, Schneider I, Bauernfeind A, Mitov I. *Dissemination of CTX-M extended spectrum beta-lactamase producing isolates of Klebsiella pneumoniae in four hospitals in Sofia and Pleven*. Probl Infect Parasit Dis. 2012; 40(1):5-9.
10. Sabtcheva S, Kantardjiev T, Ivanova M, Kaku M. *Prevalence of the fluoroquinolone-modifying acetyltransferase gene aac(6')-Ib-cr among extended-spectrum beta-lactamase-producing enterobacterial isolates in a cancer hospital in Bulgaria*. Probl Infect Parasit Dis. 2008; 36(1):21-22.

SUCCESSFUL TREATMENT OF PERITONITIS CAUSED BY GLYCOPEPTIDE-RESISTANT *ENTEROCOCCUS FAECIUM,* AND EXTENDED-SPECTRUM B-LACTAMASE-PRODUCING *ESCHERICHIA COLI, ENTEROBACTER CLOACAE*, *KLEBSIELLA PNEUMONIAE,* AND *KLEBSIELLA OXYTOCA*. CASE REPORT

B. Todorova[1*], S. Sabtcheva[1], K. Neykov[2], E. Raicheva[3], B. Tzingilev[2], V. Tabakov[2], T. Kantardjiev[4]

[1] Laboratory for Clinical Microbiology, Specialised hospital for active treatment in oncology, Sofia, Bulgaria
[2] Clinic of Urology, Specialised hospital for active treatment in oncology, Sofia, Bulgaria
[3] Intensive care unit, Specialised hospital for active treatment in oncology, Sofia, Bulgaria
[4] National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria

ABSTRACT

Background: Considering the high burden of morbidity and mortality, postoperative peritonitis is a feared complication of intra-abdominal surgery. Typically this is a polymicrobial infection and the most common isolates include aerobic Gram-negative bacilli, anaerobes, and enterococci. Here, we describe a case of postoperative complication involving multidrug-resistant pathogens in a cancer patient. **Case presentation:** 79-year-old male patient diagnosed with cancer of the bladder and prostate was admitted to the urology ward at a 242-bed oncology hospital in Sofia, Bulgaria, for radical cystoprostatectomy. Due to small bowel adhesion to the pelvis and ileus causing peritonitis, two re-operations ensued. Empirical therapy was initiated with piperacillin/tazobactam, teicoplanin, and metronidazole. The first positive abdominal drainage fluid cultures yielded ESBL-producing *Klebsiella pneumoniae* and *K. oxytoca* followed by isolation of glycopeptide-resistant *Enterococcus faecium,* and ESBL-producing *Escherichia coli*, and *Enterobacter cloacae*. Piperacillin/tazobactam was replaced with administration of meropenem and linezolid, together with metronidazole until clinical improvement and negative cultures. **Conclusion:** We report a clinical case of successful treatment of peritonitis caused by glycopeptide-resistant *E. faecium,* and ESBL-producing *E. coli, E. cloacae*, *K. pneumoniae,* and *K. oxytoca*. Revealing the evolution of underlying resistance mechanisms and changes in microbial etiology during the course of therapy will improve the management of patients undergoing surgical interventions and suffering from comorbidities.

Key words: peritonitis, glycopeptide-resistant *E. faecium,* ESBL-producing *Enterobacteriaceae*

ADDRESS FOR CORRESPONDENCE:
Bozhana Todorova,
Laboratory for Clinical Microbiology, Specialised hospital for active treatment in oncology,
6 Plovdivsko Pole Str, Sofia 1756, Bulgaria
e-mail: bojana_e@abv.bg

INTRODUCTION

Clinical presentations of intra-abdominal infections can range from localised peritonitis to diffuse inflammation of the abdominal cavity. Bowel injuries, such as perforation, strangulation, or infection are a common cause of intraperitoneal infections manifested as peritonitis (1). With its high burden of morbidity and mortality, postoperative peritonitis is a feared complication of intra-abdominal surgery. Typically this is a polymicrobial infection and the most common isolates include aerobic Gram-negative bacilli, anaerobes, and enterococci (2, 3). Empirical antimicrobial therapy relies on broad-spectrum antibiotics until culture results are obtained. Precise performance of antibiotic susceptibility assays determines the de-escalation or escalation of antibiotics (1, 4). Our aim was to present a clinical case of successful treatment of peritonitis caused by glycopeptide-resistant *Enterococcus faecium,* and extended-spectrum β-lactamase-producing *Escherichia coli, Enterobacter cloacae*, *Klebsiella pneumoniae,* and *Klebsiella oxytoca* in a cancer patient in parallel with the changing microbial etiology in the course of therapy.

CASE PRESENTATION

79-year-old male patient diagnosed with cancer of the bladder and prostate was admitted to the urology ward at a 242-bed oncology hospital in Sofia, Bulgaria, for radical cystoprostatectomy. The patient had a history of undergoing left nephrectomy and three assigned surgeries for the bladder cancer in other healthcare facilities. The radical cystoprostatectomy was performed by laparotomy along with lymph node dissection followed by difficult postoperative period. On the 6th day after the cystoprostatectomy the patient was re-operated due to complications arising from small bowel adhesion to the pelvis and ileus causing peritonitis. Six days later laparotomy was performed for the third time with anterior abdominal wall reconstruction and creation of ileostomy. In accordance with the hospital guidelines, a combination of ampicillin/sulbactam and metronidazole was administered as surgical prophylaxis during the first laparotomy. Along with the development of complications, empirical therapy was initiated with piperacillin/tazobactam, teicoplanin, and metronidazole. Two days after the second laparotomy abdominal drainage fluid cultures yielded *K. pneumoniae* and *K. oxytoca* isolates with multidrug-resistant phenotype. Species identification of isolates was determined by Vitek-2 (bioMérieux, Marcy l'Etoile, France). Susceptibility to antimicrobials was determined by the disc diffusion method following the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (5). Results were interpreted according to EUCAST breakpoints (6). Suggestive evidence of Extended-spectrum β-lactamase (ESBL) production was revealed by the double-disk synergy test (7). The *Klebsiella* strains were categorised as ESBL producers and susceptible to piperacillin/tazobactam. Abdominal drainage fluid cultures and peritoneal fluid collected on the day of the third laparotomy, yielded glycopeptide-resistant *E. faecium, E.coli*, and *E. cloacae* besides the already detected *Klebsiella* isolates. *E. coli* and *E. cloacae* were also ESBL-positive. Identical in susceptibility profile *E. faecium* strain was also recovered from stool and urine cultures. Stool sample culture yielded also significantly high *Candida albicans* counts. After obtaining the antimicrobial susceptibility results, piperacillin/tazobactam was replaced with administration of meropenem, linezolid, and fluconazole together with metronidazole until clinical improvement and negative cultures. As a usual consequence of a prolonged treatment with carbapenem antibiotics, sixteen days after the third laparotomy abdominal drainage fluid and urine cultures were positive for *Stenotrophomonas maltophilia,* treated subsequently with levofloxacin. The patient was discharged on hospital day 38 in satisfactory general condition.

DISCUSSION

Carcinoma of the bladder and prostate ranks among the most common cancers in men and its incidence increases with age. A large percentage of new cases are registered in individuals aged 75–84 years. Radical cystoprostatectomy is a complex surgical procedure and a standard treatment but it is associated with complications, morbidity, prolonged recovery time, and extended hospital stays (8, 9). Further entanglement arises from the fact that patients with this disease are typically older and often have a history of comorbidities. Therefore treatment of elderly patients with urinary bladder cancer and prostate cancer poses challenges to urologists (9).

Enterococci and members of the *Enterobacteriaceae* are part of the normal intestinal flora in humans, but they are also involved in urinary infections, bacteraemia, intra-abdominal, and nosocomial infections. Glycopeptide-resistant enterococci present a serious problem in hospital settings with very limited treatment options. In Bulgaria the first vancomycin-resistant enterococcal infections were reported in 2005 from the University Hospital in Stara Zagora (10). Cases of glycopeptide-resistant enterococci causing peritonitis successfully treated with linezolid and achieving eradication of the infection were reported in studies from Korea (11, 12). Polymicrobial infections involving ESBL-producers are well known as further impeding the antimicrobial treatment. Previous reports from Bulgarian hospitals identify different families of ESBL enzymes in *Klebsiella* and other members of the *Enterobacteriaceae*, with CTX-M-like and SHV-like often found as the most prevalent (13, 14).

In conclusion we report a clinical case of successful treatment of peritonitis caused by glycopeptide-resistant *E. faecium,* and ESBL-producing *E. coli, E. cloacae*, *K. pneumoniae,* and *K. oxytoca* in a cancer patient. Investigating the evolution of microbial etiology in the course of therapy and the underlying mechanisms of resistance will improve the management of patients undergoing surgical interventions and suffering from comorbidities.

REFERENCES

1. Jang JY, Lee SH, Shim H, Choi JY, Yong D, Lee JG. Epidemiology and Microbiology of Secondary Peritonitis Caused by Viscus Perforation: A Single-Center Retrospective Study. Surg Infect (Larchmt). 2015; 16(4):436-442.
2. Steinbach CL, Töpper C, Adam T, Kees MG. Spectrum adequacy of antibiotic regimens for secondary peritonitis: a retrospective analysis in intermediate and intensive care unit patients. Ann Clin Microbiol Antimicrob. 2015; 14:48.
3. Mosdell DM, Morris DM, Voltura A, Pitcher DE, Twiest MW, Milne RL, Miscall BG, Fry DE. Antibiotic treatment for surgical peritonitis. Ann Surg. 1991; 214:543–549.
4. Solomkin JS, Mazuski JE, Bradley JS, et al. Diagnosis and management of complicated intra-abdominal infection in adults and children: Guidelines by the Surgical Infection Society and the Infectious Diseases Society of America. Surg Infect. 2010; 11:79–109.
5. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Disk Diffusion Test Methodology. Available from: http://www.eucast.org/ast_of_bacteria/disk_diffusion_methodology/ .
6. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Breakpoint tables for interpretation of MICs and zone diameters. Available from: http://www.eucast.org/clinical-breakpoints/.
7. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Guideline for the detection of resistance mechanisms and specific resistances of clinical and/or epidemiological importance. Available from: http://www.eucast.org/resistance_mechanisms/.
8. Erlich A, Zlotta AR. Treatment of bladder cancer in the elderly. Investig Clin Urol. 2016; 57 Suppl 1:S26-35.
9. Yasui T, Tozawa K, Ando R, Hamakawa T, Iwatsuki S, Taguchi K, Kobayashi D, Naiki T, Mizuno K, Okada A, Umemoto Y, Kawai N, Sasaki S, Hayashi Y, Kohri K. Laparoscopic Versus Open Radical Cystectomy for Patients Older than 75 Years: a Single-Center Comparative Analysis. Asian Pac J Cancer Prev. 2015; 16(15):6353-6358.
10. Lazarova G, Kantardjiev T, Velinov C, Rachkova K, Rukanova D, Dukova I, Djeneva H. Vancomycin-resistant enterococci in hospitalized patients with urinary tract infections – first report in Bulgaria. Trakia J Sciences. 2005; 3(4):13-14.
11. Yang JW, Kim YS, Choi SO, Han BG. Successful use of intravenous linezolid in CAPD patient with vancomycin-resistant enterococcal peritonitis. Perit Dial Int. 2011; 31:209–210.
12. Song IJ, Seo JW, Kwon YE, Kim YL, Lim TS, Kang EW, Chang TI. Successful treatment of vancomycin-resistant enterococcus peritonitis using linezolid without catheter removal in a peritoneal dialysis patient. Perit Dial Int. 2014; 34(2):235-239.
13. Markovska R, Keuleyan E, Ivanova D, Markova B, Proevska J, Leseva M, Schneider I, Bauernfeind A, Mitov I. Dissemination of CTX-M extended spectrum beta-lactamase producing isolates of *Klebsiella pneumoniae* in four hospitals in Sofia and Pleven. Probl Infect Parasit Dis. 2012; 40(1):5-9.
14. Sabtcheva S, Kantardjiev T, Ivanova M, Kaku M. Prevalence of the fluoroquinolone-modifying acetyltransferase gene aac(6')-ib-cr among extended-spectrum beta-lactamase-producing enterobacterial isolates in a cancer hospital in Bulgaria. Probl Infect Parasit Dis. 2008; 36(1):21-22.

MULTIPLE VIRAL PATHOGENS CAUSE "UNDIFFERENTIATED" CNS INFECTIONS

I. Christova[1], I. Trifonova[1], R. Vatcheva[2], V. Ivanova[1], T. Gladnishka[1], N. Korsun[1], A. Stoyanova[1], L. Nikolaeva-Glomb[1]*

[1] National Centre of Infectious and Parasitic Diseases, Sofia, Bulgaria
[2] University Multifunctional Hospital for Active Treatment "Tsaritsa Yoanna", Sofia, Bulgaria

ABSTRACT

In an attempt to elucidate etiology, we tested 110 serum samples from patients with "undifferentiated" viral CNS infections for antibodies against herpes simplex virus (HSV), varicella zoster virus (VZV), Epstein-Barr virus (EBV), Coxsackie B viruses (CVB), influenza viruses, parainfluenza viruses, tick-borne encephalitis virus (TBE), and West Nile fever virus (WNV). A total of 21 (19.1%) of the samples were reactive showing presence of IgM antibodies against at least one of the tested viruses. The most common reactivity was found against either EBV, Coxsackie B viruses or influenza viruses – 11 (10%) of the samples reacted with one of the three viruses, followed by HSV-2, TBEV, VZV, and parainfluenza viruses – 6 (5.5%) of the samples were reactive to one of the latter four viruses. Cases of HSV-1 and WNV infections were not detected. The vast majority of the annually recorded viral meningitis and encephalitis cases in Bulgaria are diagnosed only on clinical manifestations and the diagnosis is usually not supported by detection of virus species as the etiological agent. In this study, we succeeded to identify a possible viral etiological agent in one fifth of the cases, showing that investigation for multiple virus species should be pursued in an attempt to confirm the viral etiology of the CNS infection.

Key words: meningitis, CSF, enteroviruses, influenza viruses, TBE

ADDRESS FOR CORRESPONDENCE:
Iva Christova,
Head, National reference laboratory
of vector-borne infections, leptospirosis
and listeriosis,
NCIPD,
26 Yanko Sakazov blvd,
Sofia 1504, Bulgaria

INTRODUCTION

Hundreds of "undifferentiated" viral meningitis and encephalitis cases are reported every year to the Bulgarian Ministry of Health. The term encompasses all officially registered CNS infections with clinical manifestations suggesting viral etiology but with no causative agent detected.

In an attempt to elucidate the missing etiology, a collection of serum samples from patients with "undifferentiated" viral CNS infections was tested for the presence of antibodies against herpes simplex virus (HSV), varicella zoster virus (VZV), Epstein-Barr virus (EBV), Coxsackie B viruses (CVB), influenza viruses, parainfluenza viruses, tick-borne encephalitis virus (TBE), and West Nile fever virus (WNV).

Usually, herpes simplex virus infection is the most common cause of infectious encephalitis in humans (1). HSV-1 causes up to 90% of HSV encephalitis cases. HSV-2 infection is a rare one and is implicated mostly in meningitis cases. On the other hand, recurrence of meningitis is typical for HSV-2 infection.

Varicella zoster virus (VZV) is a neurotropic virus with life-long latency in sensory ganglia. VZV can cause encephalitis, myelitis, and acute myeloradiculitis. In immunocompromised patients, VZV can induce multifocal encephalitis and necrotising myelitis.

Epstein-Barr virus (EBV) causes infectious mononucleosis. Less than 5% of patients develop meningitis, encephalitis or polyradiculomyelitis before, during or after suffering infectious mononucleosis.

All members of the *Herpesviridae* family are double-stranded DNA viruses. Herpesviruses are divided into 3 subfamilies. Alpha-herpesviruses include HSV-1, HSV-2, and VZV. Typically, they have short reproductive cycle and establish latency in sensory ganglion neurons. Beta-herpesviruses include human cytomegalovirus and the roseola viruses HHV-6 and HHV-7. They replicate relatively slowly and establish latency in multiple cell types and organs. Gamma-herpesviruses represented by EBV and HHV-8, are oncogenic. They also replicate relatively slowly but establish latency in restricted cell types such as lymphocytes. All herpesviruses establish a life-long latency

and reactivate intermittently.

Enteroviruses cause more than 90% of viral meningitis cases and only occasionally are implicated as the cause of encephalitis (2). They affect mainly children under 10 years of age. The *Enterovirus* genus belongs to the *Picornaviridae* family which consists of small, non-enveloped single-stranded RNA viruses. The multitudinous genus of enteroviruses includes 7 species that are pathogenic to humans: enterovirus A, B, C, and D and rhinovirus A, B, and C. Among them, enteroviruses A, B, C, D are those that can cause meningitis, myelitis, encephalomyelitis, Guillen-Barre syndrome, etc. The notorious EV-71 as well as several coxsackie A viruses, are included in species EV-A; Coxsackie B viruses, Coxsackie virus A9, and echoviruses belong to species EV-B; species EV-C includes polioviruses 1-3, several Coxsackie A viruses and several newer enteroviruses assigned a number; and species EV-D includes 5 serotypes, among them EV-68 (2). Enteroviral meningitis is caused primarily by echoviruses and Coxsackie B viruses. Enterovirus infection should be suspected in children with neurologic symptoms.

Besides respiratory complications, influenza viruses can also cause infections of the CNS. Development of CNS diseases is the most common extra-respiratory complication of infection with influenza A viruses. Meningitis, encephalitis, encephalopathies, myelitis can be caused by influenza A viruses. Influenza viruses belong to the *Orthomyxoviridae* family of enveloped negative-strand RNA viruses. Influenza A viruses are subtyped based on their surface glycoproteins, hemagglutinin, and neuraminidase. Seasonal, pandemic, and zoonotic influenza A viruses affect humans. Seasonal influenza A viruses cause yearly epidemics (H3N2, H1N1). Pandemic influenza viruses are a result of cross-species transmission and subsequent efficient transmission among humans (H1N1, H2N2). Zoonotic influenza virus infections are a result of zoonotic transmission with inefficient transmission among people (H7N9, H5N1).

Some viruses of the *Paramyxoviridae* family can also cause a wide range of neurological manifestations ranging from acute encephalitis to severe long-term infections. These include measles, mumps, Hendra, and Nipah viruses. Viruses in this virus family are enveloped and contain single-stranded negative-sense RNA.

Some flaviviruses typically cause infections of CNS. Tick-borne encephalitis virus and West Nile virus, even rare in this country, should also be considered. The *Flavivirus* genus of the *Flaviviridae* family includes arboviruses that cause encephalitis. Flaviviruses are single-stranded positive-sense RNA viruses. TBE viruses are classified into three subtypes – European, Siberian, and Far-Eastern subtype. TBE virus is transmitted by *Ixodes ricinus* ticks. Neurological manifestations of TBE include meningitis (50%), meningoencephalitis (40%) or meningoencephalomyelitis (10%) (3). West Nile virus is subdivided in seven genetic lineages, of which lineages 1 and 2 cause the epidemics. WNV is transmitted by *Culex* mosquitoes in an enzootic cycle between birds. Small outbreaks of WNV infections appear yearly in countries neighbouring Bulgaria and the first WNV cases were described in Bulgaria last year. It is estimated that 1 in 140 infected people develops meningoencephalitis, ranging from 1 in 50 in patients over 65 years to 1 in 300 in patients younger than 65 years.

Viruses belonging to all of the above described families can cause infections of the CNS and are likely to be found as etiological agents of "undifferentiated" viral meningitis and encephalitis cases. In the present study, serum samples from such patients were tested by ELISA to detect specific IgM antibodies against possible viral etiological agents in order to establish the extent to which each of these virus species could contribute to the etiology of the observed CNS infection.

MATERIALS AND METHODS

Patients

A total of 110 patients with clinically diagnosed viral meningitis (n=78), encephalitis (n=23) or meningoencephalitis (n=9) of unknown etiology were enrolled in the study. Serum samples from the patients were collected in The Clinical Microbiology Laboratory of University Hospital "Tsaritsa Yoanna – ISUL" (n=41), The National Reference Laboratory of Vector-Borne Infections (n=49), and The National Reference Laboratory of Enteroviruses (n=20). The serum samples were collected in the period between 2012 and 2015. The age of the patients varied between 3 and 88, mean 42.

ELISAs

All 110 serum samples from the patients were tested for antibodies against 9 different virus species using commercially available ELISA

tests (4). Test procedures and interpretation of results were carried out according to manufacturer's instructions. The serum samples were tested for infection with four herpesviruses – HSV-1, HSV-2, VZV, and EBV, infection with Coxsackie B viruses representing one of the enteroviruses, namely EV-B, for infection with two respiratory viruses – influenza A and parainfluenza viruses, and for two flavi arboviruses – TBEV and WNV. The ELISA tests used in the survey included: Anti-HSV-1 IgM ELISA with antigens of glycoprotein C1 (Euroimmun, Germany); Anti-HSV-2 IgM ELISA with antigens of glycoprotein G2 (Euroimmun, Germany); Anti-VZV Glycoprotein IgM ELISA (Euroimmun, Germany); Anti-EBV Capsid antigens IgM ELISA (Euroimmun, Germany); Anti-Coxsackie B virus IgM ELISA (Euroimmun, Germany); Anti-Influenza A and B virus pool IgM ELISA (Euroimmun, Germany); Anti-Parainfluenza viruses pool of types 1 to 4 IgM ELISA (Euroimmun, Germany); Anti-TBE Virus IgM ELISA (Euroimmun, Germany); and Anti-West Nile Virus IgM ELISA (Euroimmun, Germany). In addition, IgM reactive sera for TBE and WNV were tested for IgG antibodies.

RESULTS

Serum samples (n=110) collected within 4 years between 2012 and 2015 from patients with undifferentiated viral meningitis, encephalitis or meningoencephalitis were tested by ELISAs (one sample per patient) for infection with 9 virus species known to cause acute CNS infections.

A total of 21 (19.1%) of the samples were reactive showing presence of IgM antibodies against at least one of the tested viruses. Of them, 17 (15.5%) samples were clearly IgM positive and other 4 were IgM borderline (3.6%). One of the samples showed reactivity against 3 viruses (positive for VZV and WNV,

Table 1. Specific IgM antibodies found in serum samples from patients with acute undifferentiated viral CNS infections against 9 different virus species known to cause neurological infections.

Virus infections tested	Number of positive samples	Number of borderline samples	Total number of reactive samples (%)
HSV-1	0	0	0
HSV-2	1	1	2 (1.8%)
VZV	1	0	1 (0.9%)
EBV	4	0	4 (3.6)
Coxsackie B viruses	2	1	3 (2.7%)
Influenza virus	3	1	4 (3.6)
Parainfluenza virus	1	0	1 (0.9%)
TBE	3	0	3 (2.7%)
WNV	4	2	6 (5.5%)
TOTAL	19	5	24* (21.8%)

* Sum of positively reacted serum samples exceeds the actual number of positive samples because two of the serum samples have reacted with multiple viral pathogens.

and borderline for HSV-2) and another one showed reactivity to two of the tested viruses (EBV and Influenza virus). Summarised results are presented in Table 1. The sum of positively reacted serum samples exceeds the actual number of positive samples because two of the serum samples have reacted with multiple viral antigens.

In addition, all samples that were found IgM positive for TBE or WNV were tested for IgG antibodies to prove specificity of the reaction since IgG antibodies appear very early in the course of flavivirus infections. IgG antibodies against TBE virus were confirmed in 2 of the 3 IgM reactive sera. IgG antibodies against WNV were found in none of the 6 IgM reactive serum samples.

The most common reactivity was found against either EBV, Coxsackie B viruses or influenza viruses – 11 (10%) of the samples reacted with one of the three viruses, followed by HSV-2, TBEV, VZV, and parainfluenza viruses – 6 (5.5%) of the samples were reactive to one of these four viruses. Cases of HSV-1 and WNV infections were not detected.

All patients with reactive samples detected, were clinically diagnosed as viral meningitis except the two patients with TBEV infection detected, who were diagnosed as viral encephalitis and meningoencephalitis.

DISCUSSION

The vast majority of annually registered viral meningitis and encephalitis cases in Bulgaria are diagnosed only on clinical manifestations and lack a confirmed etiological agent. In this study, we have succeeded to identify the possible viral etiological agent in one fifth of the cases, revealing that investigation for multiple virus species as the probable cause of the disease should be pursued in an attempt to confirm the viral etiology of the CNS infection concerned.

The present study reveals that investigated "undifferentiated" viral meningitis and encephalitis cases are mostly due to complications of Ebstein-Barr, Coxsackie B, and influenza virus infections. Less common reaction is detected against HSV-2, VZV, and parainfluenza viruses.

Most of the tested serum samples originate from patients with meningitis and this is the most probable reason why HSV-2 is suspected and not HSV-1 which is known to cause encephalitis. Coxsackie B virus infections when symptomatic are mainly associated with meningitis. Development of CNS diseases is the most common extra-respiratory complication of infection with influenza viruses.

One of the serum samples reacted with VZV, HSV-2, and WNV and most probably was infected with VZV because reactivity to HSV-2 was borderline on one hand, and on the other hand, reactivity to WNV was not confirmed by detection of specific IgG antibodies. Another serum sample showed reactivity to both EBV and influenza virus and was probably infected with EBV and cross-reacted with influenza virus.

TBEV infection is limited to certain geographical regions and is a rare one in Bulgaria but still should be considered in differential diagnosis. Detection of IgG antibodies in addition to IgM antibodies against TBEV in 2 of the 3 samples has confirmed the diagnosis and clearly has shown that TBE is not so unusual in this country. The results of the present study also reveal that TBE infections are manifested as encephalitis or meningoencephalitis while the rest of the detected viral infections presented as meningitis.

Although WNV infections are extremely rare in the country, they should also be considered during the corresponding seasons when mosquitoes are active.

Using PCR to test CSF samples of patients with neurological disorders, Kleines et al (5) detected EBV in 1.6%, VZV in 1.3%, HSV in 1.24%, and EV in 0.4% of the patients. In patients with infectious central nervous system disorders, HSV, VZV, and EV prevailed (5).

Similar design of the study, but focused on patients over 65 years, was conducted by Parisi et al (6). They detected viral RNAs in 2.3% of the samples – HSV in 35.4%, EV in 23.1%, EBV in 21.5%, VZV in 18.5%, and CMV in 1.5% of the positives.

When most of the patients are children, enteroviruses considerably prevail. For example, in the study of Akhvlediani et al (7) EV was detected in 26 patients, VZV in 4, and HSV-1 in 2 patients out of 140 patients with infectious CNS disorders tested by multiplex PCR of their CSF samples. In this study, 58% of the patients were children.

The results of our study reveal that herpesviruses, enteroviruses, and influenza viruses are the most common causes of CNS infections. Appropriate testing is needed to properly identify the etiological agent in each case.

References

1. Chow FC, Glaser CA, Sheriff H, Xia D, Messenger S, Whitley R, Venkatesan A. Use of clinical and neuroimaging characteristics to distinguish temporal lobe herpes simplex encephalitis from its mimics. Clin Infect Dis 2015, 60:1377–1383. doi:10.1093/cid/civ051 25.
2. Adams MJ, Lefkowitz EJ, King AMQ, Bamford DH, Breitbart M, Davison AJ, Ghabrial SA, Gorbalenya AE, Knowles NJ, Krell P, Lavigne R, Prangishvili D, Sanfaçon H, Siddell SG, Simmonds P, Carstens EB. Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses Arch Virol 2015, 160:1837-1850.
3. Kaiser R. The clinical and epidemiological profile of tick-borne encephalitis in southern Germany 1994-98: a prospective study of 656 patients. Brain 1999, 122:2067–2078.
4. Christova I, Ivanova V, Trifonova I., T. Gladnishka, E. Taseva. Comparison of a complement fixation assay, ELISA and immunoblot for serologic diagnosis of hantavirus infections in Bulgaria. Probl Infect Paras Dis 2010, 38:47-48.
5. Kleines M, Scheithauer S, Schiefer J, Hausler M. Clinical application of viral cerebrospinal fluid PCR testing for diagnosis of central nervous system disorders: a retrospective 11-year experience. Diagn Microbiol Infect Dis 2014;80:207–15.
6. Parisi S, Basso M, Del Vecchio C, Andreis S, Franchin E, Dal Bello F, Pagni S, Biasolo Ma, Manganelli R, Barzon L, Palu G. Viral infections of the central nervous system in elderly patients: a retrospective study. Int J Infect Dis 2016, 44:8-10.
7. Akhvlediani T, Bautista CT, Shakarishvili R, Tsertsvadze T, Imnadze P, Tatishvili N, Davitashvili T,Samkharadze T, Chlikadze R, Dvali N, Dzigua L, Karchava M, Gatserelia L, Macharashvili N,Kvirkvelia N, Habashy EE, Farrell M, Rowlinson E, Sejvar J, Hepburn M, Pimentel G, Dueger E, House B, Rivard R. Etiologic agents of central nervous system infections among febrile hospitalized patients in the country of Georgia. PLoS One 2014, 9 (11):e111393. doi: 10.1371/journal.pone.011139

MEDITERRANEAN SPOTTED FEVER (MSF) WITH UNUSUAL PORTAL OF ENTRY – CASE REPORT

T. Doichinova, G. Gancheva, I. Pakov

Medical University – Pleven, Department of Infectious Diseases, Epidemiology, Parasitology, and Tropical Medicine, Bulgaria

Abstract

Background: Mediterranean spotted fever (or Boutonneuse fever) is re-emerging tick-borne infectious disease, caused by *Rickettsia conorii*. The trend of disease distribution in Bulgaria is increasing in the last two decades. Pleven region is not endemic and only sporadic cases have been registered. The aim was to report a case of *R. conorii* infection with unusual portal of entry. **Case presentation:** 58-years-old woman crushed a tick manually followed by conjunctival exposure of the left eye to the tick`s blood. Six days later there was a sudden onset of fever and shivering. Intensive conjunctival hyperaemia, swollen eyelids, and worsened vision ensued. On the 5th day after hospital admission generalised papulous rash developed involving the palms and the soles. The fever persisted, the woman was in a state of nervous excitement with headache and hepatomegaly; there were no neurological signs. Laboratory investigations revealed normocytosis with granulocytosis (WBC 6.9 x 10^9/L, granulocytes 0.76, respectively), C-reactive protein 16.1 mg/dL, fibrinogen level 5.14 g/L, and normal liver biochemical tests. Later on, the degree of leucocytosis and granulocytosis increased and the C-reactive protein rose up to 90.2 mg/l. Haemoculture was negative and serological investigation was positive for *R. conorii*. Complex etiologic and supportive treatment was administered, including ciprofloxacin, lincomycin, and metronidazole intravenously, eye-drops, and symptomatic drugs. The patient improved and discharged after twelve days of hospital treatment. **Conclusions:** The case is interesting because of the unusual portal of entry. An increased awareness of MSF could eliminate the risk of delayed and incorrect diagnosis and also improve the prognosis.

Key words: *Rickettsia conorii*, Mediterranean spotted fever, intraocular inflammation

ADDRESS FOR CORRESPONDENCE:
Tsetsa Georgieva Doichinova
Clinic of Infectious Diseases
Department of Infectious Diseases, Epidemiology, Parasitology, and Tropical Medicine at Faculty of Public Health, Medical University – Pleven, Bulgaria
8a Georgi Kochev str.
5800 Pleven, Bulgaria
Phone: +35964886439, mobile +359888729532
e-mail: doichinova@abv.bg

Mediterranean spotted fever (MSF) or Boutonneuse fever is an acute systemic re-emerging infectious disease first described in 1910 in Tunisia by Conor and Bruch. It is transmitted to humans by tick bites. The brown dog tick *Rhipicephalus sanguineus* is the usual vector. The etiologic agent is *Rickettsia conorii*, a small intracellular organism belonging to the spotted fever group of the *Rickettsia* family (1). MSF is an endemic disease occurring during spring and summer in Asia, Africa, and Mediterranean countries. MSF is the most common endemic tick-transmissible rickettsiosis in Bulgaria during the last decade and the trend of distribution is rising. Pleven region is not endemic, only sporadic cases have been registered. Our **aim** was to describe a case of MSF with unusual portal of entry. We used the records in the patient's hospital documentation.

CASE REPORT

Fifty-eight-year-old woman was admitted on 8 May, 2014 to the Clinic of Infectious Diseases at the University Hospital – Pleven. Six days prior to admission she crushed a big tick with fingers followed by exposure of her left eye to the sudden bleeding of tick blood. Immediately, hyperaemia of the conjunctiva appeared followed by swelling of the eyelids and visual worsening. On the next day, fever of 38°C with shivering, weakness, and edema of the cervical region developed. The patient reported a past ischemic brain stroke with left-side hemiparesis and hypertonic disease. She had no contact with pets.

On physical examination, the patient was afebrile, conscious, but in a state of nervous excitement and unstable walking. The skin was without rash. The left eye was with intensive hyperaemia of the conjunctiva, suffusion on the sclera and swollen eyelids. Enlarged cervical and inguinal lymph nodes were found. She presented also with a coated tongue and mild sore throat. There were no pulmonary auscultation findings. The

heart rate was normal and the blood pressure was 140/90. The abdomen was painless. Slightly enlarged liver was found without splenomegaly. There were no signs of meningeal irritation.

Laboratory tests revealed erythrocytes 4.1; 3.89; 4.44 x 10^{12}/L; haemoglobin 119; 111; 123 g/L; white-blood cell count 6.9; 7.8; 12.9 x 10^{9}/L; neutrophils 0.78; 0.80; 0.59; platelet count 225; 240; 506 x 10^{12}/L; aspartate aminotransferase 18; 33 IU/L; alanine aminotransferase 13, 35 IU/L; alkaline phosphatase 102 IU/L; gamma-glutamyl transferase 89 IU/L; fibrinogen 5.14 g/L; C-reactive protein 16.1; 90.2; 0.74 mg/L; total protein 64 g/L; albumins 39 g/L; blood urea nitrogen 3.7 mmol/L; creatinine 75 μmol/L; Na^+ 136; K^+ 4,0; Cl^- 100 mmol/L. Uroculture and haemoculture were negative. Serological investigation by ELISA was positive for MSF: IgM antibodies titer was 27 (reference value <11) and IgG antibodies 4 (reference value < 9).

The patient had consultation with ophthalmologists who recommended a local therapy.

Empirical treatment was initiated intravenously with ceftiaxone but on the fourth hospital day the characteristic generalised maculopapular rash developed, involving the palms and soles. Antimicrobial therapy was changed to ciprofloxacin and metronidazole intravenously, and local ocular treatment was performed.

The patient improved and was discharged after twelve days of hospital treatment. She was afebrile, with resolution of the rash, and the left eye was without hyperaemia and swelling.

Discussion

MSF is known as an acute disease with benign prognosis but the patients' quality of life worsens due to fever and multiple organ disorders. The disease could be life-threatening for the elderly, immune-compromised, and patients with co-morbidity (2).

Biology of the agent, epidemiology, pathogenesis, and immunogenesis are still being studied. The clinical symptoms and organ damage are continuously investigated.

Systemic disorders involve cardiovascular system – myocarditis (3), heart-rhythm disorders (4), phlebitis (5), gastrointestinal tract (granulomatous hepatitis) and bleeding (6), kidneys – tubulointerstitial nephritis (7, 8), nervous system – encephalitis, neuritis (9, 10), arthritis and myositis (11), respiratory system – bronchopulmonary symptoms, exudative pleuritis (12).

The aim of this report was to focus the attention on affecting the sense organs, and in particular, the visual sense. The prevalence of these complications is not high but with various localisations and degree of severity. There are reports on changed visual ability (13), uveitis (14, 15), optic nerve neuritis (16), keratitis (17, 18), and posterior segment damage (19).

Popivanova N (2006) performed the most extended study on MSF in Bulgaria, including variability of the clinical forms and systemic disorders. Her special attention was focused on affecting the sense organs. The author described cases with conjunctivitis and reversible visual disorders. All patients crushed ticks with fingers followed by contamination of the eyes, i.e. the conjunctiva was a portal of entry. Fever and maculopapular rash always presented without eschar ("black spot") (20). In the case we report, the patient also developed the typical rash and fever, "black spot" was absent but the infection was transmitted by direct inoculation of tick blood in the eye. We did not find in the current literature *R. conorii* entering the human body in a similar mode and this fact makes the reported case unique.

Laboratory investigations revealed the typical changes associated with moderately severe clinical form of MSF – discrete leucocytosis with granulocytosis, normal haemoglobin level, erythrocytes, and platelet number, normal liver biochemical parameters, slightly elevated fibrinogen and C-reactive protein levels (after treatment – normal), normal blood urea nitrogen, creatinine, and electrolytes.

Empirical treatment of the patient was started with ceftriaxone due to unclear diagnosis. Following the appearance of the typical rash, therapy was changed to ciprofloxacin intravenously – a consideration in accordance with the literature (20).

Conclusions: The case is interesting because of the unusual portal of entry. An increased awareness of MSF could eliminate the risk of delayed and incorrect diagnosis and also improve the prognosis.

References:

1. Chan Y, Riley S and Martinez J. *Adherence to and invasion of host cells by spotted fever group Rickettsia species.* Frontiers in Microbiology. 2010; 1:1-10.
2. Dalla P, Petala A, Maltezou H, Maltezos E, Calonge M. *Fatal Mediterranean spotted fever in Greece.* Clin Microbiol Infect. 2010; 16:589–592.
3. Bellissima P, Bonfante S, La Spina G, Turturici M, Bellissima G, Tricoli D. *Complications of mediterranean spotted fever.* Infez Med. 2001; 9(3):158-62 (in Italian).
4. de Groot R, Oranje A, van der Heyden A, Vuzevski V, Schaap G, van Joost T. *Rickettsia conorii infection complicated by supraventricular tachycardia in a ten year-old child. Acta Leiden.* 1984; 52:45-52.
5. Gómez-Mateos J, Lazano de León F, Pineda J, Díaz-Torres M. *Phlebitis of the legs: a complication of Mediterranean bouton-*

neuse fever. Med Clin (Barc). 1986; 87(5):218.
6. Scaffidi A, Furitano G, Scaffidi L. *Complications of boutonneuse fever.* Minerva Med. 1981;72(31):2053-62.
7. Galicia M, Fort J, de Torres I, Camps J, Piera L. *Tubulointerstitial nephritis and Mediterranean spotted fever.* Nephron. 1991; 58(1): 128.
8. Galicia M, Fort J, Moliner E, de Torres I. *Renal involvement in Mediterranean boutonneuse fever.* Med Clin (Barc). 1987; 88(20):830 (in Spanish).
9. Botelho-Nevers E, Foucault C, Lepidi H, Brouqui P. *Cerebral infarction: an unusual complication of Mediterranean spotted fever.* Eur J Intern Med. 2005; 16(7):525-7.
10. Alioua Z, Bourazza A, Lamsyah H, Erragragui Y, Boudi O, Karouach K, et al. *Neurological feature of Mediterranean spotted fever: a study of four cases.* Rev Med Interne. 2003; 24(12): 824-9 (in French).
11. Behar D, Ben-Ami H. *Myositis accompanying Rickettsia conorii infection.* Isr Med Assoc J. 2001; 3(6):471-2.
12. Raoult D, Jean-Pastor M, Xeridat B, Garnier J, Weiller P, Garcin G, et al. *Mediterranean boutonneuse fever. Apropos of 154 recent cases.* Ann Dermatol Venereol. 1983; 110(11):909-14.
13. Granel B, Serratrice J, Rey J, Conrath J, Disdier P, Weiller P. *Impaired visual acuity in Mediterranean boutonneuse fever.* Presse Med. 2001; 30(17):859 (in French).
14. Pinna A, Sechi LA, Serru A, Zanetti S, Fadda G, Carta F. *Endogenous panuveitis in a patient with Rickettsia conorii infection.* Acta Ophthalmol Scand. 2000 ; 78(5):608-9.
15. Agahan A, Torres J, Fuentes-Páez G, Martínez-Osorio H, Orduña A. *Intraocular inflammation as the mainmanifestation of Rickettsia conorii infectio.* Clin Ophthalmol. 2011; 5:1401–1407.
16. Castanet J, Costet C, Dubois D, Lacour J, Ortonne J. *Optic neuropathy in Mediterranean boutonneuse fever.* Presse Med. 1988;17(9):439-40.
17. Alio J, Ruiz-Beltran R, Herrera I, Artola A, Ruiz-Moreno JM. *Rickettsial keratitis in a case of Mediterranean spotted fever.* Eur J Ophthalmol. 1992; 2(1):41-3.
18. Alió J, Ruiz-Beltran R, Herrero-Herrero J, Hernandez E, Guinaldo V, Millan A. *Retinal manifestations of Mediterranean spotted fever.* Ophthalmologica. 1987; 195(1):31-7.
19. Khairallah M, Ladjimi A, Chakroun M, Messaoud R, Yahia S, Zaouali S, et al. *Posterior segment manifestations of Rickettsia conorii infection.* Ophthalmology. 2004; 111(3):529-34.
20. Popivanova N. Mediterranean spotted fever (Marseillean fever). Monograph. Plovdiv, 2006 (in Bulgarian).

CASE REPORT WITH PULMONARY AND NEURAL TUBERCULOSIS

G. Gancheva, T. Doichinova, I. Pakov

Medical University – Pleven, Department of Infectious Diseases, Epidemiology, Parasitology, and Tropical Medicine, Bulgaria

ABSTRACT

Background: Neurotuberculosis is the most hazardous type of systemic tuberculosis (TB) because of high mortality and possible serious neurological complications. Early diagnosis and prompt treatment are crucial for favourable outcome. **Case report:** A 36-year-old female patient was admitted to suburban Infectious Diseases Ward with a one-week history of fever, headache, and vomiting. Primary diagnosis was "viral encephalitis". Four days later, she became unconscious and was transported to the Clinic of Infectious Diseases at the University Hospital – Pleven. On the physical examination, there was syndrome of meningeal irritation, depressed tendon reflexes, and a positive Babinski's sign bilaterally. Investigation of cerebrospinal fluid (CSF) revealed increased protein level (3.15 g/L), leucocytes count 80/μL (30% neutrophils and 70% mononuclears), decreased glucose level (0.61 mmol/L). *Mycobacterium tuberculosis* was confirmed by culture of CSF. CT-scan revealed brain edema and subarachnoid cyst suboccipitally. The first X-ray of the lungs was considered as "negative", the second as „pleuropneumonia". Tuberculostatic (streptomycin, isoniazid, rifampicin, pyrazinamide) and supportive treatment was performed but the patient's condition worsened, oculomotor and abducens nerves were involved, respiratory disorders appeared requiring mechanic ventilation. The patient died on the 20th day after admission. On autopsy, infiltrative-pneumonic TB, fibrinous-purulent pneumonia, and bronchiolitis were found. Subsequently tuberculous meningoencephalitis was found, visualised morphologically by lymphocytic basal meningitis and parenchymal vessel vasculitis with microthrombosis. Cortical and basal multifocal ischemia and pulmonary disorders were the direct cause of the lethal outcome. ***Conclusion:*** The globally increased incidence of TB and co-existence of extra-neural tuberculosis and neurotuberculosis require diagnostic improvement and specific therapy even in suspected cases.

Key words: tuberculosis, meningoencephalitis, pulmonary tuberculosis, tuberculostatic therapy

ADDRESS FOR CORRESPONDENCE:
Tsetsa Georgieva Doichinova
Clinic of Infectious Diseases
Department of Infectious Diseases, Epidemiology, Parasitology, and Tropical Medicine at Faculty of Public Health, Medical University – Pleven, Bulgaria
8a Georgi Kochev str.
5800 Pleven, Bulgaria
Phone: +35964886439, mobile +359888729532
e-mail: doichinova@abv.bg

INTRODUCTION

The global increase in the incidence of tuberculosis (TB) is a health issue of universal concern. The acquired immunodeficiency syndrome (AIDS) and the problem of multidrug-resistant TB (MDRTB) are among the factors that have contributed to this increase (1, 2, 3). Advanced age, alcoholism, drug abuse, poverty, malnutrition, transmigration, lymphoma, and immunosuppressive medication also contribute to increased susceptibility (4). A primary TB manifests as pulmonary TB, miliary TB, and CNS TB (5, 6). Involvement of the central nervous system (CNS) by TB is the most hazardous type of systemic TB because of its high mortality rate and possible serious neurological complications and sequels. Co-existence of extra-neural tuberculosis is reported among 50% of cases of neurotuberculosis in the literature which may be a clue to the diagnosis of CNS TB (7, 8). A tuberculous infection of CNS can present either as basal exudative meningitis or as localised tuberculoma.

Tuberculous meningitis (TBM) is the most common presentation of CNS TB accounting for 70-80% of cases (4). The granulomatous infection of the leptomeninges is characterised by a thick exudate affecting the basal parts of the brain. Probably, early meningeal exudate arises from rupture into the subarachnoid space of a microscopic granuloma. This lesion is due to haematogenous dissemination of distant tuberculous focus or is a result of rupture of tuberculoma or miliary tubercle. It can become active even years after initial infection (7).

TBM most often presents with fever, headache, decreased level of consciousness, and meningeal signs such as neck stiffness, photophobia, and

vomiting (9, 10). The disease usually evolves gradually over two to six weeks. Convulsions are possible during the course of the illness. Involvement of cranial nerves is seen in 17-70% of patients with TBM (4). Cranial nerves II, III, IV, VI, VII, and VIII are most frequently affected either due to ischemia of the nerve or its nucleus, or due to compression from basal exudates. Without treatment, death usually occurs in three to six weeks and with treatment, mortality rates range from 6-27% (7).

We report a case with co-existence of extra-neural tuberculosis (pulmonary TB) and neurotuberculosis (tuberculous meningoencephalitis).

CASE REPORT

Thirty-six-year-old female patient with a one-week history of fever, headache, and vomiting was admitted to suburban Infectious Diseases Ward with diagnosis “viral encephalitis” after obtained informed consent. On admission, she was without mental alterations and the complaint was strong occipital headache. Four days later, she became disoriented and was transported to the Clinic of Infectious Diseases at the University Hospital because of worsening. Six months prior to admission, she had contact with relatives suffered from TB and three months later she was treated for “pneumonia”.

On the physical examination she demonstrated marked stiffness, positive Kernig’s sign, depressed patellar and Achilles’ reflexes, and positive Babinski’s sign. Cranial nerves were intact. On auscultation, decreased breathing without crackles was found with normal heart frequency and blood pressure.

Laboratory investigations revealed white-cells count in blood 11.800/mm^3 and neutrophils 76%. Lumbar puncture was performed and CSF investigation revealed protein level 3.15 g/L and leucocytes 80/μL (30% neutrophills and 70% mononuclears). Glucose in CSF was 0.61 mmol/L. Both, direct microscopy and culture of CSF were positive for *Mycobacterium tuberculosis*. CT scan of the head revealed brain edema and subarachnoid cyst suboccipitally. According to the neurosurgeon this was congenital cyst in cysterna magna and the left cerebellar hemisphere that did not need surgical treatment. The first X-ray of the lungs was concluded as “negative” (Figure 1), and the second – as „pleuropneumonia” (Figure 2).

The treatment started with quadruple tuberculostatic combination of streptomycin, isoniazid, rifampicin, and pyrazinamide. Supportive treatment was performed with dexamethasone, mannitol, furosemide, hepatoprotectors, infusion of fluids and blood products. Nevertheless, the patient’s condition worsened, cranial nerves were involved (oculomotor nerve and abducens nerve palsies), respiratory disorders appeared requiring mechanic ventilation. The patient died on the 20th day after admission to the hospital.

On autopsy, infiltrative-pneumonic TB in the three right pulmonary lobes, as well as fibrinous-purulent pneumonia and purulent bronchiolitis in the medial and inferior lobe were found and confirmed histologically. Subsequently, tuberculous meningoencephalitis was found, morphologically visualised by lymphocytic basal meningitis and parenchymal vessel vasculitis with microthrombosis. The direct cause of the lethal outcome was multifocal cortical and basal ischemia with severe pulmonary disorders.

DISCUSSION

We present here a case of severe tuberculous meningoencephalitis co-existing with extra-neural pulmonary TB. The sudden onset of strong headache, vomiting, and fever is not typical for neurotuberculosis. The rapid unfavourable course of the case also is not characteristic of CNS TB – within four days following admission the patient became unconscious.

Suggestive for diagnosis is the fact that six months prior to admission, the patient had contact with relatives suffered from TB and three months later she was treated for “pneumonia”. Probably at that time was the onset of pulmonary TB.

The diagnosis of tuberculous meningitis is difficult. According to Thwaites et al (2002), there are five diagnostic criteria such as patient’s age, duration of complaints, number of WBC count, number of leucocytes in CSF, and percentage of neutrophils in CSF. Total diagnostic index (DI) is calculated according to the formula: DI (age) + DI (blood white-cell count) + DI (history of illness) + DI (cerebrospinal fluid white-cell count) + DI (% neutrophils in cerebrospinal fluid). Therefore the suggested diagnostic rule is: if the patient has a total diagnostic index score of 4 or less, he or she has tuberculous meningitis, and if the patient has a score of more than 4, he or she has bacterial meningitis (11). In the case we report here, the total diagnostic index score was -3 and was suggestive for tuberculous meningitis (Table 1).

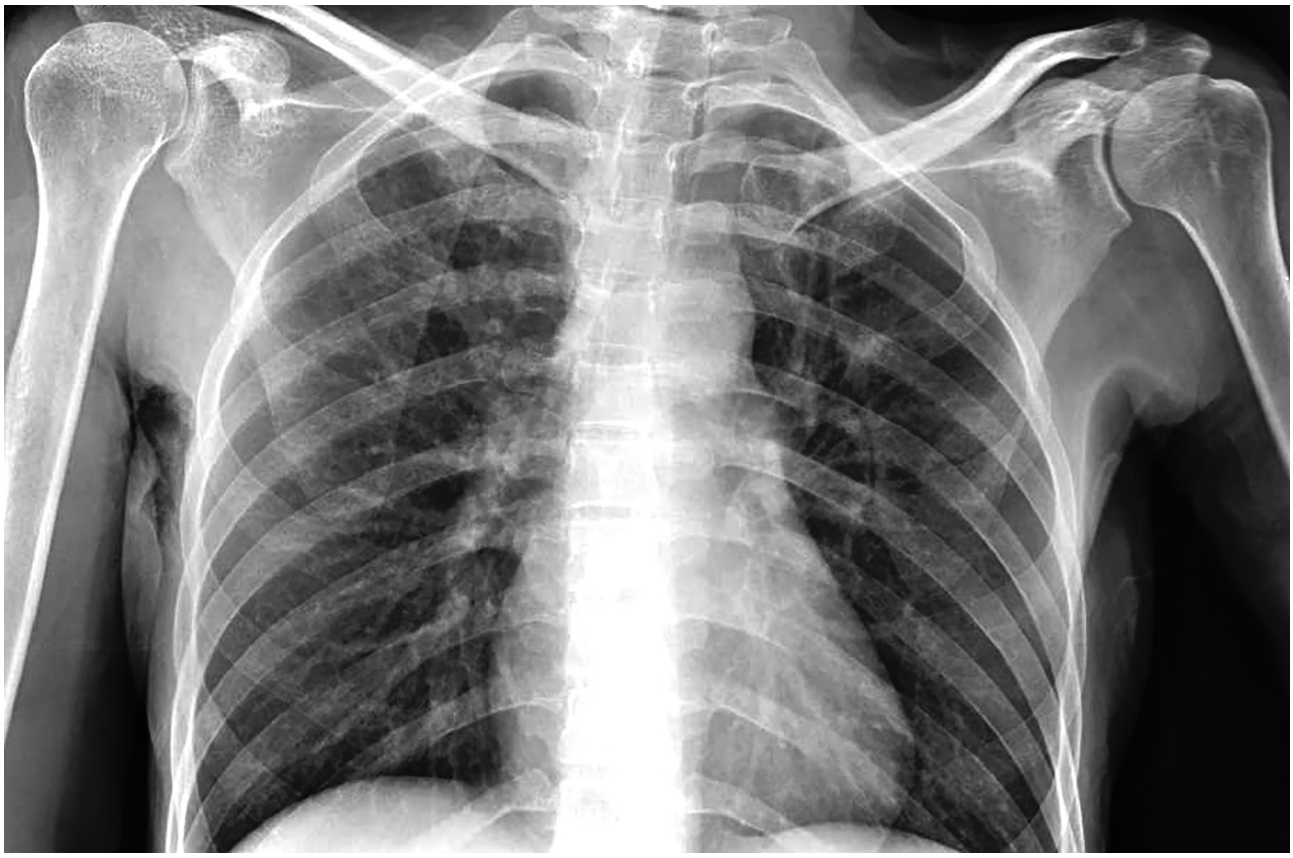

Figure 1. Pulmonary X-ray of the reported case on admission.

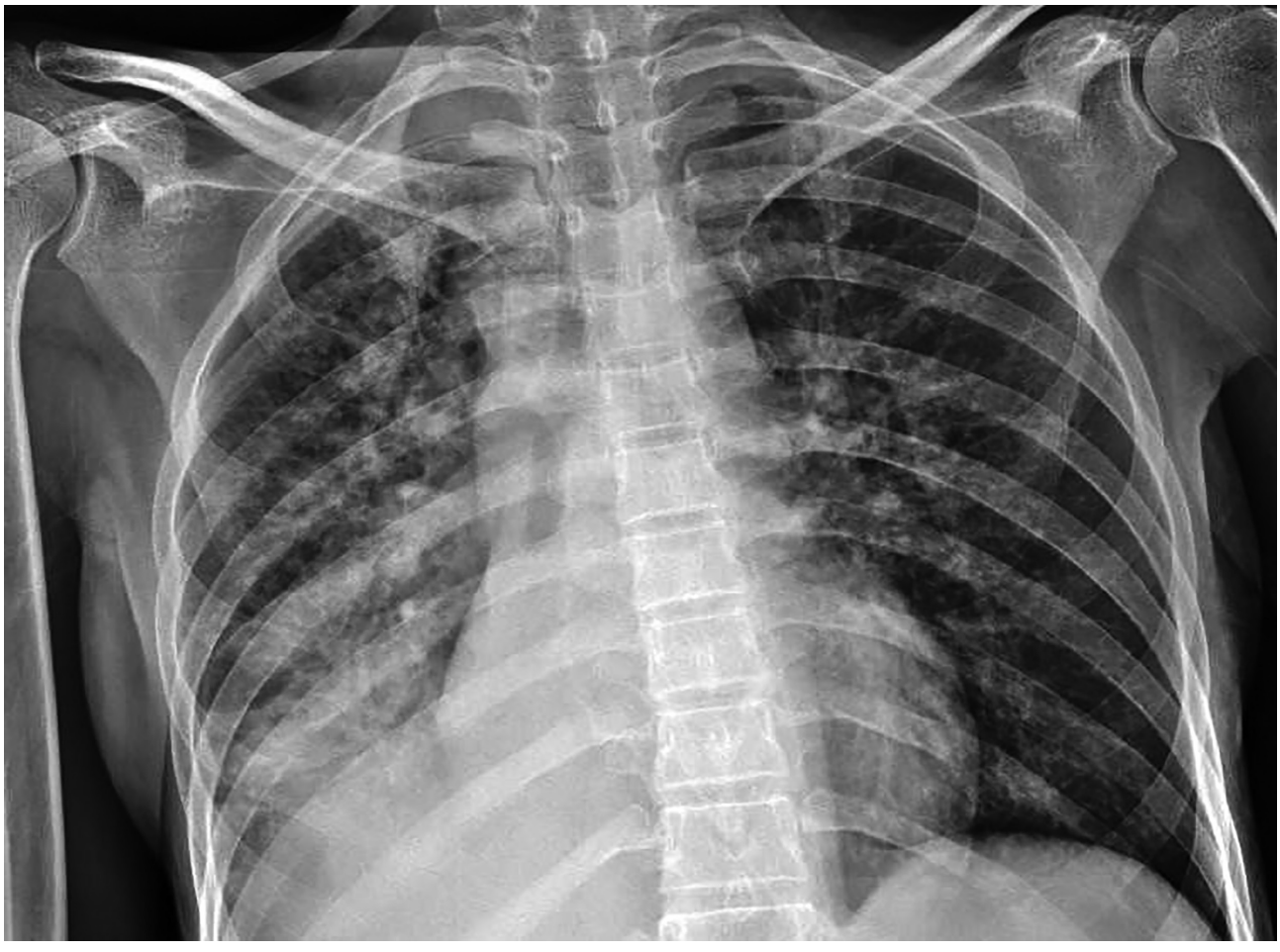

Figure 2. Pulmonary X-ray of the reported case thirteen days after admission.

Table 1. Diagnostic assessment of the reported case according to Thwaites' diagnostic index (6).

Characteristic	Value	Index	Patient's characteristic	Patient's index
Age	≥36 <36	2 0	36	2
WBC	>15,000/mm^3 <15,000/mm^3	4 0	11,800/mm^3	0
History duration	<6 days ≥6 days	0 -5	7 days	-5
Leucocytes in CSF	>900/mm^3 <900/mm^3	3 0	80/mm^3	0
Percentage of neutrophils in CSF	≥75 <75	4 0	30	0
Total DI				**-3***

WBC – white blood cells; DI – diagnostic index
*Total diagnostic index less than 4 is suggestive for tuberculous meningitis

Qamar et al (2013) considered that features predictive of tuberculous meningitis diagnosis are as follows: protein/glucose ratio of ≥2, pleocytosis < 800, and presence of hydrocephalus (12). In the case we report, mild leucocytosis was found and CSF changes suggested typical tuberculous constellation – markedly elevated protein level contrasted with mild pleocytosis, neutrophils/mononuclears ratio of 3/7, and low glucose level. The ratio CSF protein/CSF glucose level was 5.16 and in accordance with the consideration of Qamar et al (2013) (12). Dendane et al (2013) considered six features predictive of tuberculous meningitis diagnosis: female gender, duration of symptoms, presence of localising signs, blood leucocytes count, level of serum sodium, and pleocytosis in CSF (13).

The subarachnoid cyst found by CT also provoked diagnostic difficulties. We did not find reports on the simultaneous presence of such formation and tuberculous infection of CNS. In our case CT findings should be interpreted in a complex approach with results from other investigations – an opinion mentioned by Botha et al. (2012) (14). At the same time, pulmonary X-ray investigation is helpful for diagnosis but in the first X-ray there were no characteristic tuberculosis findings. The second X-ray investigation revealed typical pulmonary TB but the patient's condition irreversibly worsened irrespective of initiated treatment with four tuberculostatic drugs.

The treatment was in concordance with contemporary principles of antituberculous therapy mentioned in the WHO Global Tuberculosis Programme (2010) (15). Unfortunately, simultaneous presence of pulmonary TB and tuberculous meningoencephalitis worsened the prognosis of the presented case. Delayed diagnosis of previously untreated pulmonary TB also contributed to the lethal outcome.

Conclusion: The globally increased incidence of TB and co-existence of extra-neural tuberculosis and neurotuberculosis require diagnostic improvement and specific therapy even in suspected cases.

CONFLICT OF INTEREST STATEMENT (AUTHORS):

We certify that there is no conflict of interest with any financial organisation regarding the material discussed in the manuscript.

REFERENCES:

1. Woldeamanuel Y, Girma B. *A 43-year systematic review and meta-analysis: case-fatality and risk of death among adults with tuberculous meningitis in Africa.* J Neurol. 2014; 261(5):851-65.
2. Yordanova S, Bachiyska E, Atanasova Y, Todorova Y, Baikova A, Kantardjiev T. *Multidrug resistant tuberculosis in Bulgaria-microbiological aspects*. Probl Inf Parasit Dis. 2013; 41(1):5-8.

3. Bergval I, Sengstake S, Bachiyska E, Brankova N, Tankova K, Levterova V, Ivanova A, Atanasova Y, Yordanova S, Abadía E, Sola C, Akhalaia M, Aspindzelashvili R, van Soolingen D, Anthony R, Kantardjiev T, Panaiotov S. *Evaluation of a new molecular test for the identification of drug resistance in mycobacterium tuberculosis clinical isolates*. Probl Inf Parasit Dis. 2012; 38(1): 22-25.
4. Radhakrishnan K, Kishore A, Mathuranath PS. *Neurological tuberculosis.* In: Sharma SK, ed. Tuberculosis. 1st ed. New Delhi: Jaypee Brothers; 2001:209-228.
5. Radev M. *Tuberculosis.* In: Infectology. Academic Press "M. Drinov", 2001:253-261 (in Bulgarian).
6. Genev G. *Tuberculous meningitis.* In: Therapy of infectious diseases. Press "Medicine and physical culture", 2011:273-275 (in Bulgarian).
7. Gauba C, Varma M. *Tuberculosis of the central nervous system.* Apollo Med. 2005; 2:21-8.
8. Atanasova Y, Bachiyska E, Yordanova S, Todorova Y, Baykova A, Kantardjiev T. *Rapidly growing mycobacteria in suspected for tuberculosis pacients in Bulgaria.* Probl Inf Parasit Dis, 2014; 4(1):14-16.
9. Pehlivanoglu F, Yasar K, Sengoz G. *Tuberculous meningitis in adults: a review of 160 cases.* Sci World J. 2012:169028.
10. Galimi R. *Extrapulmonary tuberculosis: tuberculous meningitis new developments.* Eur Rev Med Pharmacol Sci. 2011; 15(4):365-86.
11. Thwaites G, Chau T, Stepniewska K, Phu N, Chuong L, Sinh D, et al. *Diagnosis of adult tuberculous meningitis by use of clinical and laboratory features.* Lancet. 2002; 360(9342):1287-92.
12. Qamar F, Rahman A, Iqbal S, Humayun K. *Comparison of clinical and CSF profiles in children with tuberculous and pyogenic meningitis; role of CSF protein: glucose ratio as diagnostic marker of tuberculous meningitis.* J Pak Med Assoc. 2013; 63(2):206-10.
13. Dendane T, Madani N, Zekraoui A, Belayachi J, Abidi K, Zeggwagh A, et al. *A simple diagnostic aid for tuberculous meningitis in adults in Morocco by use of clinical and laboratory features.* Int J Infect Dis. 2013; 17(6):e461-5.
14. Botha H, Ackerman C, Candy S, Carr J, Griffith-Richards S, Bateman K. *Reliability and diagnostic performance of CT imaging criteria in the diagnosis of tuberculous meningitis.* PLoS One 2012; 7(6):e38982.
15. WHO Global Tuberculosis Programme. Treatment of Tuberculosis: Guidelines for National Programmes. WHO Press, Switzerland, 108 (2010).

CONFLICT OF INTEREST STATEMENT (AUTHORS)

I certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

Author name Date Signature

When there is conflict of interest, specify the company title and the relationship with the Author.

CONFLICT OF INTEREST STATEMENT (REVIEWERS)

I certify that have no personal or financial conflict of interest with authors of the manuscript provided me for review.

Reviewer name Date Signature

When there is conflict of interest, please specify the relationship with the Author.

STATEMENT ABOUT PROTECTION OF HUMAN SUBJECTS
AND ANIMALS IN RESEARCH

I certify that this study involving human subjects is in accordance with the Helsinky declaration of 1975 as revised in 2000 and that it has been approved by the relevant institutional Ethical Committee.

Author name Date Signature

I certify that this study involving animals followed the institutional and national guide for the care and use of laboratory animals.

Author name Date Signature

