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Romanian Archives of Microbiology and Immunology

IN VITRO ACTIVITY OF TIGECYCLINE AND IMIPENEM EVALUATED UNDER AEROBIC AND ANAEROBIC CONDITIONS

C.S. Stîngu, F. Bethmann, P. Eisermann, S. Wendt, A.C. Rodloff

Institute of Medical Microbiology and Epidemiology of Infectious Diseases, Leipzig, Germany

ABSTRACT

The aim of this study was to evaluate and compare the *in vitro* activity of imipenem and tigecycline against Gram positive and Gram negative isolates, using 2 commercially available Etest strips under aerobic and anaerobic conditions.

194 prospectively collected clinical strains representing 11 different species, were used in this study. All the strains were tested for their susceptibility to tigecycline (Etest strips from Liofilchem, Italy) and imipenem (Etest strips from bioMerieux, France and Liofilchem, Italy) under aerobic and anaerobic conditions. Differences between the two Etest strips could be determined for *Escherichia coli*, *Enterobacter* spp. when incubated anaerobically and *Klebsiella pneumoniae* aerobically and anaerobically. Differences between aerobic and anaerobic cultivation were also observed in almost all cases. Still, the predicted category was in agreement in all experiments.

Keywords: tigecycline, imipenem, Etest

REZUMAT

Scopul acestui studiu a fost să evalueze şi să compare acţiunea *in vitro* a imipenemului şi tigeciclinei asupra izolatelor gram pozitive şi gram negative incubate aerob şi anaerob, folosind E teste de la două firme: BioMerieux (Franţa) şi Liofilchem (Italia).

194 de tulpini reprezentând 11 specii diferite au fost colectate prospectiv pentru acest studiu. Toate tulpinile au fost testate aerob şi anaerob la tigeciclină (E test Liofilchem) şi imipenem (E test Liofilchem si BioMerieux). Diferenţe în ceea ce priveşte valorile CMI (concentraţia minimă inhibitorie) citite folosind cele 2 tipuri de E teste au fost găsite pentru *Escherichia coli, Enterobacter* sp. în anaerobioză şi pentru *Klesbiella pneumoniae* atât în aerobioză, cât şi în anaerobioză. Au fost de asemenea găsite diferenţe în ceea ce priveşte valorile CMI citite aerob şi anaerob. Totusi, nu au existat diferenţe privind încadrarea în categoriile rezistent, intermediar şi sensibil.

INTRODUCTION

Tigecycline and imipenem are important antimicrobial agents for the treatment of serious infections, therefore correct MIC (minimum inhibitory concentration) determination is important. Imipenem is one of the therapeutic options against extended spectrum ß lactamase (ESBL) producing Gram negative bacilli (GNB) and tigecycline, one of the remaining options against extensively drug resistant (XDR) GNB [1, 2, 3, 4, 5]

While tigecycline Etest strips from bioMerieux and Liofilchem have been compared with each other [6], there are neither studies that investigated the effect of aerobic or anaerobic cultivation on the MICs, nor studies that compared the imipenem commercially available Etest strips from bioMerieux and Liofilchem.

MATERIAL AND METHODS

194 prospectively collected clinical strains representing 11 different species were screened in this study. All the clinical strains had been collected from patients from the University Hospital Leipzig from November 2013 until April 2014 and were identified using the VITEK-MS system (bioMerieux) operated on the V2.0 Knowledge Base for clinical use.

The following bacterial strains were examined: *Haemophilus influenzae* (n = 12), *Streptococcus pneumoniae* (n = 15), *Acinetobacter baumannii* (n = 14), *Serratia* spp. (n = 10), *Escherichia coli* (n = 27), *Enterobacter* spp. (n = 25), *Pseudomonas aeruginosa* (n = 21), *Staphylococcus aureus* (n= 26), *Streptococcus agalactiae* (n = 10), *Enterococcus* spp. (n = 15), *Klebsiella* spp. (n = 25), *Stapylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922.

The standardized inoculum was prepared by suspending bacteria (precultured for 16 h at 36 ± 0.5 ºC) with sterile 0.9 % NaCl solution to adjust the turbidity to that of McFarland density no. 0.5. All the tests were performed in duplicate.

Mueller-Hinton agar (*E. coli, Enterobacter* spp, *Serratia* spp, *Klebsiella* spp, *A. baumannii*,

**Corresponding author:* PD Dr. habil Cătălina-Suzana Stîngu, Institute of Medical Microbiology and Epidemiology of Infectious Diseases, Liebigstr. 21, 04103, Leipzig, Germany, tel: 00493419715242, fax: 00493419715209, CatalinaSuzana.Stingu@medizin.uni-leipzig.de

Pseudomonas spp., *S. aureus* and *Enterococcus* spp.), chocolate agar (*H. influenzae*) and Haemophilus Test Medium (HTM) (*H. influenzae*), Mueller-Hinton blood agar (*S. pneumoniae, S. agalactiae*) were incubated aerobically and anaerobically (Whitley MG 1000, anaerobic workstation, Meintrup Laborgeräte GmbH, Lähden-Holte, Germany) at 37°C for 24 hours, except for obligate aerobes *Pseudomonas* and *Acinetobacter*, which were not tested under anaerobic conditions. Tigecycline Etest strips were provided by Liofilchem® and the Imipenem Etest strips by Liofilchem® and bioMerieux.

All the susceptibility testing and interpretations were conducted according to EUCAST (European Committee on Antimicrobial Susceptibility Testing) Guidelines [7]. MIC_{50} and MIC_{90} were calculated for all tested strains.

RESULTS

The MIC values displayed by the reference strains were in the EUCAST range [7].

Table 1 displays the MIC`s distribution for tigecycline determined by the two different testing conditions. All the tested strains with the exception of the *P. aeruginosa*, showed *in vitro* susceptibility against tigecycline under both aerobic and anaerobic conditions. The majority of *P. aeruginosa* strains (15/19) showed intrinsically resistance against tigecycline. *Streptococcus pneumoniae*, *Serattia* spp. *Acinetobacter baumannii*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Enterococcus* strains showed no differences in MIC's under the aerobic and anaerobic conditions. *Enterobacter* spp. and *Klebsiella* spp. showed elevated median MIC's (one and respectively two fold dilutions) under the aerobic conditions. *Escherichia coli* strains showed no differences for the median MIC´s values but MIC_{90} was a dilution higher under anaerobic conditions. The incubation atmosphere does not significantly influence the MIC values of tigecycline against *H. influenzae*, regardless of the type of media used. On the other hand, a difference of two fold dilution in MIC's values of tigecycline was observed between the chocolate agar and HTM agar (the election media recommended by EUCAST) regardless the incubation atmosphere. The MIC values of tigecycline were elevated (two fold dilution) when the tests were performed using the chocolate agar.

Tabel 2 displays the MIC's distribution for imipenem determined aerobically and anaerobically using Etest strips from bioMerieux and Liofilchem. Again for the majority of the tested strains (*S. pneumoniae*, *Serratia* spp. *A. baumannii*, *S. aureus*, *S. agalactiae*, *Enterococcus* spp.) there was no difference in MIC's between the aerobic and anaerobic conditions. *Enterobacter* spp. *Klebsiella* and *E. coli* showed again elevated MIC values (mostly one dilution) when incubated aerobically. Interestingly, although the MIC_{50} and MIC_{90} showed the same values regardless the Etest strips used, the individual MIC values were generally one dilution higher when Etest strips from BioMerieux were used. *H. influenzae* strains showed elevated MIC's when incubated aerobically (one fold dilution). The MIC values of imipenem were generally elevated (one or two fold dilutions) when the tests were performed using the chocolate agar, regardless the E test strips used. No discordances between the MIC's were observed for the two types of E test strips.

DISCUSSION

Antimicrobial susceptibility testing is performed using phenotypic methods for the establishment of the MIC. This allows that the microorganism is categorized as sensible, intermediate and resistant to the substance screened. For every species there is a given standard regarding media, inoculum, incubation, reading and interpretation of the MIC using the breakpoints tables. Any change of procedure could lead to false MIC values. The gold standard method to determine MIC is broth microdilution according to the ISO standard 201776-1 (2006). Etest is a well established and cost effective method for antimicrobial susceptibility testing in laboratories around the world. It combines the diffusion method with the generation of MICs across more than 15 dilutions. All important antibiotics are now available as Etest strips. EUCAST does not recommend a particular manufacturer of media or gradient tests but the EUCAST standards must be applied for each test. Meanwhile, there are many manufacturers that are producing such Etest strips, some of them being less expensive and therefore preferred. To our knowledge there are neither studies that investigated the effect of aerobic or anaerobic cultivation on the MICs, nor studies that compared the imipenem commercially available Etest strips from bioMerieux and Liofilchem.

The incubation atmosphere and the type of media had influenced the MIC values when both

***Table 1. In vitro* activities of tigecycline (Liofilchem® MIC Test Strips) against various bacteria (MIC distribution and $MIC_{50/90}$)**

Organism (No. of isolates)	MIC (µg/ml)										MIC_{50} µg/ml	MIC_{90} µg/ml
	<0.06	0.06	0.125	0.25	0.5	1	2	4	>4	No growth/ *not evaluable		
***H. influenzae* on Chocolate agar (n=12)**												
A			1	3	8						0.5	0.5
AN			4	5	2					1	0.25	0.5
***H. influenzae* on HTM (n=12)**												
A		3	8	1							0.125	0.125
AN	4	3	3							2	0.06	0.125
***S. pneumoniae* (n=15)**												
A	13	2									<0.06	0.06
AN	13									*2	<0.06	<0.06
***A. baumannii* (n=14)**												
A	6	4	3	1							0.06	0.125
AN										14	-	-
***Serratia* spp. (n=10)**												
A					8	2					0.5	1
AN			1	3	6						0.5	0.5
***E. coli* (n=27)**												
A		1	25	1							0.125	0.125
AN		1	18	8							0.125	0.25
***Enterobacter* spp. (n=25)**												
A					23	1	1				0.5	0.5
AN		3	6	13	3						0.25	0.5
***Pseudomonas aeruginosa* (n=21)**												
A							1	4	16		>4	>4
AN											-	-
not tested												
***S. aureus* (n=26)**												
A	3	10	13								0.06	0.125
AN	1	9	16								0.125	0.125
***S. agalactiae* (n=10)**												
A		1	9								0.125	0.125
AN		1	9								0.125	0.125
***Enterococcus* spp. (n=15)**												
A	3	8	4								0.06	0.125
AN	2	7	6								0.06	0.125
***Klebsiella* spp. (n=25)**												
A			3	8	11	2	1				0.5	1
AN			13	7	3	2					0.125	0.5

Legend: BM: bioMerieux. LF Liofilchem. A: aerobic. AN: anaerobic

Table 2. In vitro **activities of imipenem (Etest® by bioMérieux and Liofilchem® MIC Test Strips) against various bacteria (MIC distribution and $MIC_{50/90}$)**

Organism (No. of isolates)	MIC (µg/ml)												
	<0.125	0.125	0.25	0.5	1	2	4	8	16	>16	No growth/ *not evaluable	MIC_{50} µg/ml	MIC_{90} µg/ml
H. influenzae **on Chocolate agar (n=12)**													
ABM			1	2	6	1	2					0.5	2
A LF		1	1	1	6	2	1					1	2
AN BM	1	1	5	4							1	0.25	0.5
AN LF	1	1	1	6	2						1	0.5	1
H. influenzae **on HTM (n=12)**													
ABM		4	5	2	1							0.25	0.5
A LF			5	4	1	2						0.5	2
AN BM	3	4	3								2	0.125	0.25
AN LF	2	2	5		1						2	0.25	0.25
S. pneumoniae **(n=15)**													
ABM	15											<0.125	<0.125
A LF	15											<0.125	<0.125
AN BM	12										*3	<0.125	<0.125
AN LF	12										*3	<0.125	<0.125
A. baumannii **(n=14)**													
ABM	1	9	3			1						0.125	0.25
A LF	2	6	5			1						0.125	0.25
AN BM											14	-	-
AN LF											14	-	-
Serratia **spp. (n=10)**													
ABM		2	8									0.25	0.25
A LF	1	1	8									0.25	0.25
AN BM	2	4	4									0.125	0.25
AN LF	3	4	3									0.125	0.25
E. coli **(n=27)**													
ABM	4	21	2									0.125	0.125
A LF	9	17	1									0.125	0.125
AN BM	12	15										0.125	0.125
AN LF	22	5										<0.125	0.125
Enterobacter **spp. (n=25)**													
ABM		8	16		1							0.25	0.25
A LF	3	8	11	2	1							0.25	0.5
AN BM	7	16	2									0.125	0.125
ANLF	17	7		1								<0.125	0.125

(Table 2. continuation)

Organism (No. of isolates)	MIC (µg/ml)												
	<0.125	0.125	0.25	0.5	1	2	4	8	16	>16	No growth/ *not evaluable	MIC_{50} µg/ml	MIC_{90} µg/ml
***Pseudomonas aeruginosa* (n=21)**													
ABM			1	4	5	4				7		2	>16
A LF			1	3	5	4	1			7		2	>16
AN BM											21	-	-
AN LF											21	-	-
***S. aureus* (n=26)**													
ABM	25			1								<0.125	<0.125
ALF	25			1								<0.125	<0.125
AN BM	26											<0.125	<0.125
AN LF	26											<0.125	<0.125
***S. agalactiae* (n=10)**													
ABM	10											<0.125	<0.125
A LF	10											<0.125	<0.125
AN BM	10											<0.125	<0.125
AN LF	10											<0.125	<0.125
***Enterococcus* spp. (n=15)**													
ABM	1			6	5	1	1			1		1	4
A LF	1			3	8		2			1		1	4
AN BM	1		1	8	2	1	1			1		0.5	2
AN LF	1		1	7	3	1	1			1		0.5	4
***Klebsiella* spp. (n=25)**													
ABM	1	19	5									0.125	0.25
A LF	10	12	3									0.125	0.25
AN BM	15	9	1									<0.125	0.125
AN LF	22	2	1									<0.125	0.125

Legend: BM: bioMerieux. LF Liofilchem. A: aerobic. AN: anaerobic

BioMerieux and Liofilchem Etest strips were used. Some of the species tested like *Enterobacter* spp. and *Klebsiella* spp. showed elevated median MIC's under the aerobic conditions (0.25 versus 0.125 and 0.125 versus <0.125, respectively) while *E. coli* strains showed no differences for the median MIC´s values but MIC_{90} was a dilution higher under anaerobic incubation (0.125 versus <0.125). The MIC values of both tigecycline and imipenem were elevated when the tests were performed using the chocolate agar. Chocolate agar is a widely used media for cultivation of fastidious bacteria but not recommended as standard media for antimicrobial susceptibility testing. The predicted category (susceptible/intermediate and resistant) was in agreement in all experiments but MIC values

obtained using the Etest strips from both manufacturers gave variable results, some values with one or two dilutions disagreement. There are only a few recent comparative studies on the *in-vitro* antibiotic activity under different incubation atmospheres, although such experiments have been carried out since the 1970s. Verklin *et al.* compared the MICs of various facultative anaerobic enterobacteria for gentamicin, tobramycin, cephalothin, cefazolin and cefamandole [8] and found that the required amount of aminoglycosides for growth inhibition of most enterobacteria was 4 to 20 times higher under anaerobic incubation conditions than under aerobic conditions. On the other hand, no alteration of the effectiveness of cephalosporins regarding different incubation atmospheres could be demonstrated. The conclusion was even that standard MIC testing does not reflect the real conditions at the site of infection; there are significant notable differences in the efficacy of antibiotics in terms of the presence of oxygen. Ring *et al.* were able to confirm these MIC differences between aerobic and anaerobic incubation conditions [9]. In a recent study [10] it was shown that the tetracycline and spectinomycin susceptibility for *H. influenzae* decreased under anaerobic conditions. Similarly, the effectiveness of rifampicin and ciprofloxacin decreases with lowering oxygen content. The most dramatic effect was seen on the susceptibility to trimethoprim: under anaerobic growth condition the drug loses antibiotic effectiveness against *H. influenzae*. Keren *et al.* also found that the norfloxacin MIC shifts one step down in *E. coli* incubating in anaerobic atmosphere [11]

In conclusion differences between the two Etest strips could be determined for *Escherichia coli*, *Enterobacter* spp when incubated anaerobically and *Klebsiella pneumoniae* aerobically and anaerobically. Differences between aerobic and anaerobic cultivation were also observed in almost all cases. Still, the predicted category was in agreement in all experiments.

It remains necessary that all the laboratories perform internal quality control of each procedure whenever they introduce in their routine Etest strips from a new manufacturer.

Our results underline the importance of susceptibility testing according to EUCAST guidelines. Any deviation from these guidelines (e.g. change of incubation conditions) could lead to false MIC values.

Conflict of interest: The authors declare that there is no conflict of interest.

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THE PREVALENCE OF SOME EXTENDED SPECTRUM BETA-LACTAMASE GENES IN CLINICAL *PSEUDOMONAS AERUGINOSA*

Fereshteh Satarian[1], Abbas Ali Imani Fooladi[2], Seyed Fazlollah Mousavi[3], Hamid Sedighian[2], Hamideh Mahmoodzadeh Hosseini[2], Mohsen Amin[4], Fereshteh Shahcheraghi[3]

[1]*Department of Microbiology, Science & Research branch, Islamic Azad University, Tehran, Iran*
[2]*Applied Microbiology Research Center, Systems Biology and poisonings Institute Baqiyatallah University of Medical Sciences, Tehran, Iran*
[3]*Department of Bacteriology & Research Center of Microbiology, Pasteur Institute of Iran, Tehran, Iran*
[4]*Department of Drug and Food Control, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran*

ABSTRACT

Pseudomonas aeruginosa (*P. aeruginosa*) is an important nosocomial pathogen due to resistance to various antibiotics, causing high mortality rate in severe infections. In this research, we aimed to investigate the prevalence of some extended spectrum beta-lactamase (ESBL) genes and multidrug-resistant MDR properties among *P. aeruginosa* clinical isolates. 110 *P. aeruginosa* strains were isolated from clinical samples. Antimicrobial resistance pattern and ESBL production of each isolate were assessed using the disk diffusion method and phenotypic confirmatory test (PCT), respectively.

Polymerase chain reaction (PCR) was used to investigate the frequency of the genes bla_{PER-1}, bla_{GES-1}, bla_{CTX-M}, bla_{OXA-1}, bla_{OXA-4}, bla_{OXA-10} and bla_{VEB-1}. 14.54% of the isolates were phenotypically ESBL producers. Out of 110 *P. aeruginosa* strains, 80 (70.8%), 21 (18.6%), 26 (23%), 23 (20.4%), 10 (8.8%), were resistant to ciprofloxacin, ceftazidime, gentamicin, ceftriaxone and amikacin, respectively. 22 out of 110 isolates (20%) were MDR.

According to PCR results, 25 (23.1%), 51(47.2%), 8 (7.4%), 22 (20.4%), 36 (31.9%), 42 (37.2%) and 69 (61.1%) isolates out of 110 harbored the *bla*OXA-1, bla_{OXA-4}, bla_{OXA-10}, bla_{VEB-1}, bla_{PER-1}, bla_{GES-1} and bla_{CTX-M} genes, respectively. Frequency of antibiotic resistance, MDR and ESBL genes were high in the isolates. Therefore, ESBL producer isolates were screened using the combined disc diffusion test as PTC is recommended to limit dissemination of ESBLs and to reduce the mortality and morbidity rates of *Pseudomonas* infections.

REZUMAT

Pseudomonas aeruginosa (*P. aeruginosa*) este un important agent patogen nosocomial, din cauza rezistenţei sale la diverse antibiotice, producând o rată ridicată a mortalităţii în cazul infecţiilor severe. Scopul acestui studiu este investigarea prevalenţei unor gene pentru sinteza betalactamazelor cu spectru extins (BLSE) şi a proprietăţilor de multirezistenţă (MR) la izolatele clinice de *P. aeruginosa.* Au fost izolate din probe clinice 110 tulpini de *P. aeruginosa.* Au fost evaluate profilul de rezistenţă antimicrobiană şi producerea de BLSE a fiecărui izolat, utilizând metoda disc difuziei şi, respectiv, testul fenotipic de confirmare.

Reacţia de polimerizare în lanţ (PCR) a fost utilizată pentru a investiga frecvenţa genelor bla_{PER-1}, bla_{GES-1}, bla_{CTX-M}, bla_{OXA-1}, bla_{OXA-4}, bla_{OXA-10} şi bla_{VEB-1}. 14,54% dintre izolate au fost producătoare de BLSE. Din 110 tulpini de *P. aeruginosa,* 80 (70.8%), 21 (18.6%), 26 (23%), 23 (20.4%), 10 (8.8%), au fost rezistente la ciprofloxacin, ceftazidim, gentamicin, ceftriaxonă şi, respectiv, amikacină. 22 din 110 izolate (20%) au fost MR.

Potrivit rezultatelor PCR, 25 (23,1%), 51(47,2%), 8 (7,4%), 22 (20,4%), 36 (31,9%), 42 (37,2%) şi 69 (61,1%) de izolate din cele 110 purtau genele bla_{OXA-1}, bla_{OXA-4}, bla_{OXA-10}, bla_{VEB-1}, bla_{PER-1}, bla_{GES-1} şi, respectiv, bla_{CTX-M}. Atât profilul de multirezistenţă, cât şi genele pentru BLSE au fost detectate la nivele înalte în izolate. De aceea, este recomandat screening-ul izolatelor producătoare de BLSE prin combinarea testului disc difuzimetric cu cel confirmatoriu fenotipic, pentru a limita diseminarea acestor tulpini şi pentru a reduce astfel ratele de mortalitate şi morbiditate associate cu *Pseudomonas*.

Keywords: *Pseudomonas aeruginosa*, ESBLs , nosocomial infection, multidrug-resistance

1. INTRODUCTION

P. aeruginosa is an opportunistic pathogen, causing nosocomial infections in long-term hospitalization especially in cases suffering from burns and immune suppression [1].

Unfortunately, the mortality rate of such infections is so high that, in part, is attributed to the intrinsic resistance traits of the organism to various common antimicrobial agents. Multidrug- resistant (MDR) or pan-drug-resistant pathogens are on the rise, and this is the case particularly in the resistance against β-lactams [2, 3]. Pan-drug resistance resulted from the production of different hydrolyzing enzymes has spread ESBLs and is becoming a major

*Corresponding author: Abbas Ali Imani Fooladi, Applied Microbiology Research Center, Systems Biology and poisonings Institute Baqiyatallah University of Medical Sciences, Tehran, Iran, Email: imanifouladi.a@bmsu.ac.ir or imanifouladi.a@gmail.com, Tel/Fax: +98 21 88039883

concern of the health care systems worldwide [4]. In recent years, the presence of three classes of β-lactamases, A, B and D has been identified in *P. aeruginosa.* These strains are among the main pathogenic agents responsible for lethal infections in health care centers, particularly in intensive care units [5]. ESBLs, the plasmid mediated enzymes, can be transferred horizontally causing antibiotic resistance in the recipient microorganism [6]. In the past few years, four classes of ESBLs (classes A-D) have been identified in *P. aeruginosa* based on the type of substrates the enzymes use or the agents used to inhibit the enzymes [7, 8]. Class A ESBL is further divided into different subgroups including PER-1, VEB, GES, SHV, TEM and IBC which are found in certain geographical regions [7]. It has been shown that the production of PER-1 is associated with increased resistance to ceftazidime, monobactams and cephems [9]. PER-1 has atypical substrate profile, and both clavulanic acid and tazobactam can inhibit its function [10]. It is found that PER-1 is a common ESBL recovered from clinical isolates. According to previous reports from Iran, the prevalence of PER-1 varies between 17 to 27.5% among *P. aeruginosa* clinical isolates [11, 12].

GES-1 was reported in France for the first time. The expression of this enzyme confers resistance to expanded-spectrum cephalosporins consisting tazobactam, clavulanic acid and imipenem [13]. This enzyme is widely isolated from different countries such as Brazil [14] and South Africa [15]. There are no reports on detecting this gene in Iran. Although most of the ESBLs belong to TEM and SHV groups, new non-TEM non-SHV ESBL lineage have been explored. The new isolate was named CTX-M and appeared to be the main type of ESBL in various parts of the world [16].

The OXA type (class D) is the most predominant β-lactamase, which was acquired via plasmid or mobile genetic elements and has a high rate of sequence variation from 16 to 99% [17]. Class D ESBL is further divided into five subgroups including *blaOXA-group I* (*blaOXA-5,7,10,13*), *blaOXA-group II* (*blaOXA-2,3,15,20*), *blaOXA-group III* (*blaOXA-1,4,30,31*), *blaOXA-group IV* (*blaOXA-9*) and *blaOXA-group V* (LCR-1). Oxacillinase, the enzyme encoded by OXA type genes, is accountable for a remarkable resistance to cephalosporins, carboxypenicillins, etc [18].

Veb-1 gene belongs to class A and resistance to cephems and ceftazidime could depend on the expression of this enzyme [9, 19].

Few studies were designed to evaluate the frequency of some genes belonging to ESBL classes in Iran. However, most of them were focused on specific clinical samples consisting in burns or samples from infected children. In this study, we attempted to assess the distribution pattern of bla_{OXA-10}, bla_{OXA-1}, bla_{OXA-4}, bla_{VEB-1}, bla_{PER-1}, bla_{GES-1} and bla_{CTX-M} among *P. aeruginosa* strains isolated from a variety of clinical specimens in Tehran, Iran.

2. MATERIALS AND METHODS

2.1. Bacterial Isolates

A total of 110 *P. aeruginosa* strains were randomly collected from clinical specimens (wounds, blood, tracheal aspirate, sputum, ear, chest tube, bronchoalveolar lavage (BAL), bone, urine, pleural fluid and stool) from one of the educational hospitals in the north of Tehran, Iran. The isolated strains were confirmed by conventional standard biochemical tests, including oxidase, catalase, Voges-Proskauer (VP), fermentation of sugars, citrate, indole, TSI, growth at 42°C, pigment production, MR, motility on SIM media and particular odor [20]. *P. aeruginosa* ATCC 27853 was used as control.

2.2. Antibiotic Susceptibility Testing

To identify the antibiotic resistance pattern of each isolate, the disk diffusion method was used and the results were interpreted in accordance with the guidelines of Clinical and Laboratory Standards Institute(CLSI) (21). Five antibiotics, including ceftriaxone (30 µg), ceftazidime (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg) and amikacin (30 µg) were tested. All antibiotics were produced by Mast, England.

To screen for the ESBL-producing isolates, the combined diffusion method was performed on Mueller-Hinton agar using ceftazidime (30µg) and cefotaxime (30 µg) alone and in combination with clavulanic acid (10 µg) (Mast, England). The inhibition zone around combined and single discs was measured and rising ≥5 mm of a zone around clavulanic acid in comparison to zone around ceftazidime disc and cefotaxime disc alone intended to be ESBL [21].

2.3. DNA Extraction And PCR

PCR was performed to conduct a survey for the presence of bla_{VEB-1}, bla_{OXA-1}, bla_{OXA-4}, bla_{OXA-10}, bla_{PER-1}, bla_{GES-1} and bla_{CTX-M} genes as representative ESBL genes among the isolates. Total genomic DNA was extracted using the Genomic DNA Extraction kit (Bioneer, Korea) in accordance with the manufacturer's instructions.

Table 1 **- The sequence of primers**

Gene	Forward primer	Reverse primer
Bla_{PER-1}	ATGAATGTCATTATAAAAGC	AATTTGGGCTTAGGGCAGAA
Bla_{GES-1}	ATGCGCTTCATTCACGCAC	CTATTTGTCCGTGCTCAGG
Bla_{CTX-M}	CGCTTTGCGATGTGCAG	ACCGCGATATCGTTGGT
Bla_{VEB-1}	CGACTTCCATTTCCCGATGC	GGACTCTGCAACAAATACGC
Bla_{OXA-1}	AGCCGTTAAAATTAAGCCC	CTTGATTGAAGGGTTGGGCG
Bla_{OXA-4}	TCTTCAACGGATATCTCTACTGT	TGCTTTATCCCATTTGAATATGGT
Bla_{OXA-10}	TCAACAAATCGCCAGAGAAG	TCCCACACCAGAAAAACCAG

1 µl of each DNA was amplified in 25 µl of mixture reaction consisting of 10X reaction buffer, 0.1 mM of the deoxynucleoside triphosphates (dNTPs), 2 mM MgCl2, 10 ρmol of designed primers and 1 U of Taq DNA Polymerase (Cinnagen, Tehran, Iran). Specific primers are listed in Table 1. PCR was done by Eppendorf asterCycker (Hamburg, Germany) with an initial denaturation step of 5 min at 94°C; 35 cycles of 45s at 94°C, 45s at 55°C, as an annealing temperature and 45s at 72°C followed by 5 min final extension at 72°C. For evaluating the presence of desired amplicon, agarose gel electrophoresis was carried out on 1.5% agarose, stained by ethidium bromide and then products were visualized by UV transilluminator (Kiagene, Tehran, Iran).

P. aeruginosa KOAS, *K. pneumoniae* 7881, *K. pneumoniae* CHU-BICETRE harboring bla_{PER-1}, bla_{CTX-M} and bla_{GES-1}, respectively were used as the positive PCR controls.

2.4. Statistical Analysis

To compute the correlation between the incidence of genes, the drug resistance and the demographic properties of patients, we performed Chi square and Fisher's exact test using the SPSS version 15 software package (Chicago, IL). *P* value less than 0.05 was considered as statistically significant.

3. RESULTS

In this study, we surveyed the prevalence of *P. aeruginosa* harboring three ESBL genes bla_{VEB-1}, bla_{OXA-1}, bla_{OXA-4}, bla_{OXA-10}, bla_{PER-1}, bla_{GES-1} and bla_{CTX-M}, in the clinical isolates. Out of 110 strains, 30 (27.3%), 27 (24.5%), 20 (18.3%), 12 (10.9%), 4 (3.6%), 5 (4.5%), 3 (2.7%), 3 (2.7%), 3 (2.7%), 2 (1.8%) and 1 (0.9%) were isolated from wounds, bronchoalveolar lavage fluid**,** urine, sputum, pleural fluid, blood, stool, trachea, ear, chest tube and bone samples, respectively. 64 (58.2%) strains were isolated from male patients and the remaining strains were obtained from female subjects (41.8%). The ages of our cases were between 2 and 84 years, and the median age was 48.58. Most cases (29.1%) belonged to the age group of 41-50 years and only a small percentage of cases (1.8%) were over 80 years of age.

3.1. Antibiotic Susceptibility Test

To identify antibiotic resistance of each strain, the antimicrobial susceptibility test was performed. To determine the antibiotic sensitivity pattern of each strain, the antimicrobial susceptibility test was performed. Out of 110 strains, 80 (70.8%), 21 (18.6%), 26 (23%), 23 (20.4%), 10 (8.8%), were resistant to ciprofloxacin, ceftazidime, gentamicin, ceftriaxone and amikacin, respectively. 22 (20%) out of 110 isolates were MDR (resistant to more than 3 antibiotics). Our findings revealed that 14 (12.4%) and 8 (7.1%) out of 110 isolates were the ESBL positive phenotype for ceftazidime/clavulanic acid and cefotaxime/clavulanic acid, respectively. 6 (5.45%) isolates were positive for both phenotypes. Furthermore, the pattern of resistance to each antibiotic was similar in both positive and negative ESBL groups.

No statistically significant association was shown between the resistance pattern of antibiotics, multidrug-resistant properties, ESBL phenotypes and the demographic characteristics consisting of age, sex and the isolate type among the patients.

3.2. Distribution Of ESBL Genes

The frequency of bla_{VEB-1}, bla_{OXA-1}, bla_{OXA-4}, bla_{OXA-10}, bla_{PER-1}, bla_{GES-1} and bla_{CTX-M} genes was checked using PCR. An illustrative example of PCR reaction for the identification of three genes is depicted in Fig. 1. The size of the PCR products from amplifying the bla_{VEB-1}, bla_{OXA-1}, bla_{OXA-4}, bla_{OXA-10}, bla_{PER-1}, bla_{GES-1} and bla_{CTX-M} genes were 643 bp, 909 bp, 216 bp, 277 bp, 927 bp, 863 bp and 550bp, respectively. 22 (20.4%), 25 (23.1%), 51 (47.2%), 8 (7.4%), 36 (31.9%), 42 (37.2%) and

***Table 2* - The prevalence of bla_{VEB-1}, bla_{OXA-1}, bla_{OXA-4}, bla_{OXA-10}, bla_{PER-1}, bla_{GES-1} and bla_{CTX-M} genes among the resistant isolates according to different antibiotics**

Antibiotic resistance / Gene	*Ciprofloxacin* n=80 (100%)	*Ceftriaxone* n=23 (100%)	*Amikacin* n=10 (100%)	*Gentamicin* n=26 (100%)	*Ceftazidime* n=21 (100%)	*MDR* n=10 (100%)
bla_{GES-1}	32 (40)	9 (39)	3 (30)	12 (46)	10 (47.6)	8 (36.4)
bla_{CTX-M}	50 (62.4)	12 (52.2)	7 (70)	15 (57.7)	11 (52.4)	11 (50)
bla_{PER-1}	30 (37.5)	10 (43.5)	3 (30)	12 (46.2)	9 (42.9)	8 (36.4)
bla_{VEB-1}	6 (7.5)	18 (78.26)	3 (30)	7 (26.92)	4 (19.04)	5 (50)
bla_{OXA-1}	6 (7.5)	17 (73.91)	3 (30)	8 (30.76)	4 (19.04)	6 (60)
bla_{OXA-4}	8 (10)	8 (34.78)	4 (40)	10 (38.46)	10 (47.61)	9 (90)
bla_{OXA-10}	2 (2.5)	5 (21.74)	1 (10)	3 (11.53)	0 (0)	2 (20)

69 (61.1%) of 110 isolates harbored the bla_{VEB-1}, bla_{OXA-1}, bla_{OXA-4}, bla_{OXA-10}, *blaPER-1*, bla_{GES-1} and bla_{CTX-M} genes, respectively.

Table 2 summarizes the distribution of bla_{PER-1}, bla_{GES-1} and bla_{CTX-M} genes among the resistant isolates. The highest number of bla_{PER-1} positive strains was isolated from the BAL (33.3%) and wound (25%) samples (Fig. 2). Furthermore, no bla_{PER-1} gene was detected in the stool and chest tube samples. The bla_{PER-1} gene existed in 21 (32.8%) males and 15 (32.6%) female subjects.

Most of the bla_{GES-1} positive and bla_{CTX-M} positive strains were found in the BAL (31% and 23.2%, respectively) and wound (28.6 and 29%, respectively) samples (Fig. 2). None of the isolates recovered from ear, chest tube, tracheal aspirate and bone samples possessed the bla_{GES-1} gene. Moreover, none of the tracheal aspirates contained bla_{CTX-M}. Strains obtained from 27 (42.2%) males and 15 (32.6%) females harboured the bla_{GES-1} gene. 42 (65.6%) males and 27 (58.7%) females had the bla_{CTX-M} gene. No significant association was observed between the presence of the examined genes and ESBL phenotype. There was also no relationship between antibiotic resistance and the demographic characteristics including age, sex and the isolate type among the patients (*pv> 0.05).*

Most of the *blaOXA-1* positive strains were found in the BAL (Fig. 2). None of the isolates recovered from ear, stool, tracheal aspirate, sputum and bone samples possessed the *blaOXA-1* gene. Moreover, none of the stool, chest tube and sputum isolates contained *blaOXA-4.* The *blaOXA-10* gene was recovered only from wound, urine, pleural fluid, BAL, chest tube and sputum. Strains obtained from 15 (23.8%) males and 10 (22.2%) females harbored the *blaOXA-1* gene. 31 (49.2%) males and 20 (44.4%) females had the *blaOXA-4* gene. In

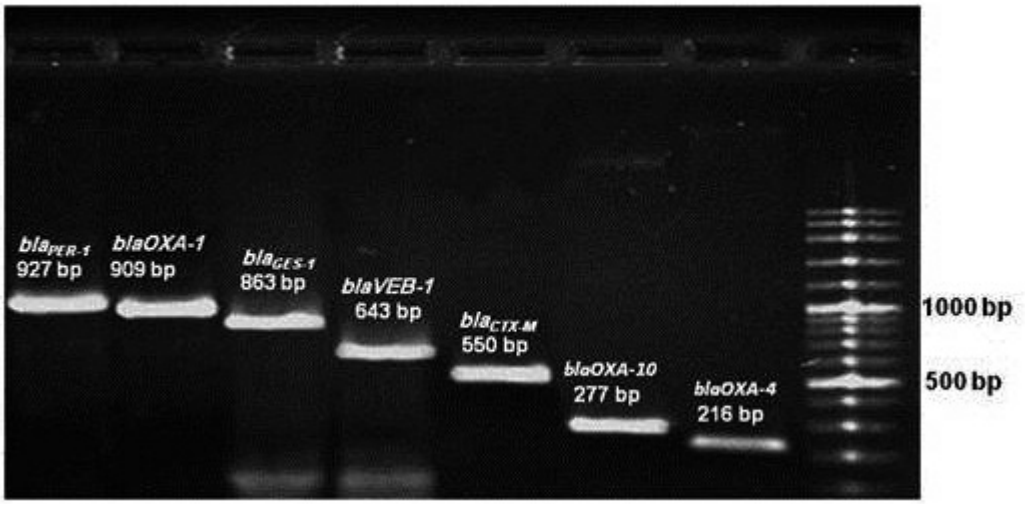

Fig. 1 - Agarose gel electrophoresis of the PCR products from the bla_{VEB-1}, bla_{OXA-1}, bla_{OXA-4}, bla_{OXA-10}, bla_{PER-1}, bla_{GES-1} and bla_{CTX-M} genes

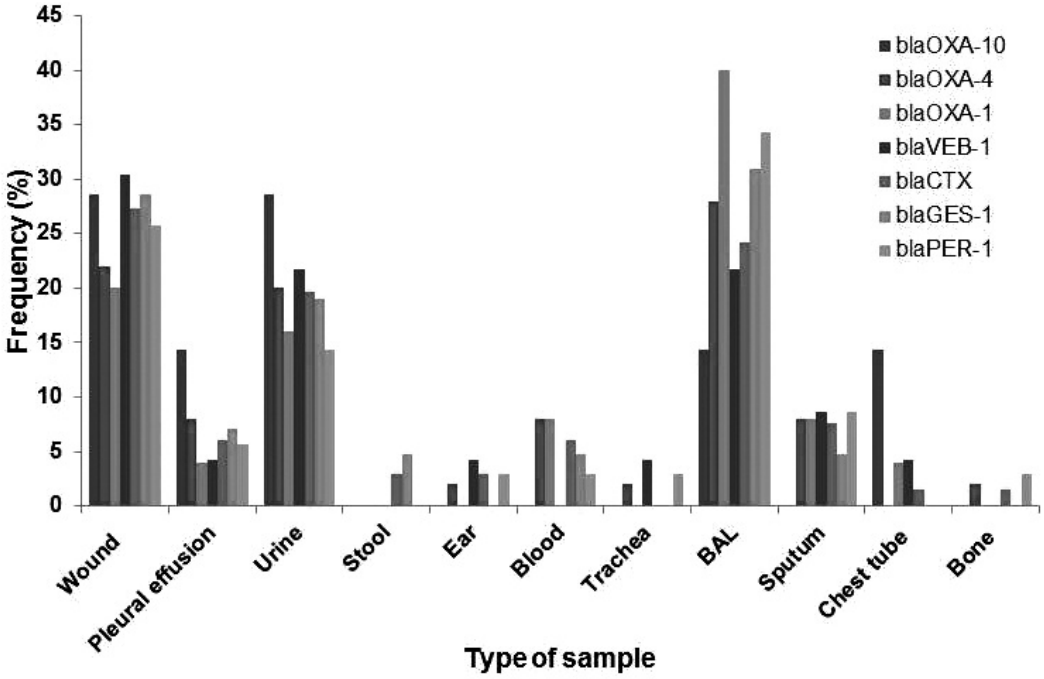

Fig. 2 - Agarose gel electrophoresis of the PCR products from the *bla*$_{VEB-1}$, *bla*$_{OXA-1}$, *bla*$_{OXA-4}$, *bla*$_{OXA-10}$, *bla*$_{PER-1}$, *bla*$_{GES-1}$ and *bla*$_{CTX-M}$ genes

addition, the *blaOXA-10* gene was identified in 3 (4.8%) males and 5 (11.1%) female subjects. No significant difference was observed between the presence of these genes and the age, sex, ESBL phenotype and source of clinical samples (*pv< 0.05).* The lower rate of resistance to gentamicin was statistically significant among isolates possessing *the blaOXA-10* gene compared to the isolates lacking this gene *(pv< 0.05).* Moreover, strains without the *blaVEB-1* gene were significantly more resistant to ciprofloxacin in comparison to the population having this gene *(pv< 0.05)*.

4. DISCUSSION

ESBLs along with MDR properties have noticeably emerged in recent years. It is reported that nosocomial bacteria harboring these genes (e.g. *P. aeruginosa*) are capable of causing severe infections. The emergence of increased resistance to antibiotics such as cephalosporins is a result of improper administration and arbitrary consumption of antibiotics [22]. On the other hand, putative resistance due to the presence of plasmid and chromosomal genes is commonplace in *P. aeruginosa* [23]. In the current study, the rate of ESBL-producing strains, MDR properties and their genetic pattern in 110 clinical isolates were evaluated. We found 12.4% ESBL-producing strains, which is more than the one reported in the United Kingdom (3.7%) [24] and Belgium (2.2%) [25]. However, the frequency of the strains in our findings is lower than the one reported in Asian countries: India (20.27%) [26], Iraq (25%) [5], Pakistan (35.8%) [27], and nearly similar to the observation in the south of Iran, Zahedan (13.3%) [16]. It is believed that the variation in findings among different countries is due to the efficiency of their infection-control policies.

The spread of the MDR strains is one of the major problems of health agencies all around the world. Our results showed an increasing rate of resistance to some third-generation cephalosporins used routinely in the treatment of *P. aeruginosa*. The antibiotic susceptibility tests revealed an increased incidence of MDR strains (20%) that was lower than an existing report from Iran (87.05%) [7]. However, findings of many studies from India, Italy, Poland, Turkey, Greece and Korea are consistent with the current research [7, 28,29-31]. The prevalence of ceftazidime resistance in our study was 31.11%, which was lower than the reports from India (53.17%) [32], Brazil (78.6%) [33] and several previous reports from Iran (57%, 66% and 100%) [7, 22] but higher than those from Iraq (29.3 %) [5] and a report from Tehran, Iran (24%) [11]. The resistance rate to amikacin, ciprofloxacin and gentamicin was 8.8%, 20.4% and 23%, respectively. These figures were lower than the ones in India [32] and Brazil

[33]. However, the data reported by Shahcheraghi *et al.* demonstrated a higher incidence of resistance to amikacin (23%) and gentamicin (31%) and a lower frequency of resistance to ceftriaxone in comparison with our findings [11].

In this study, we remarkably found high rates of *blaPER-1, blaGES-1* and *blaCTX-M* genes among our patients. The low frequency of *blaPER-1* has been reported in many countries throughout the world, such as Turkey, France, Italy and Belgium [9, 34, 35]. Findings from previous Iranian studies done by Shakibaie *et al*. [22] and Shahcheraghi *et al*. [11] showed a lower rate of *blaPER-1* (4.1% and 17%, respectively). In another Iranian study on the ESBL-producing strains isolated from burns, 49.25% of the strains possessed *blaPER-1* which is more prevalent than the strains obtained in our study [7]. Furthermore, Fazeli and his collaborators found that there is a lack of *blaPER-1* among specimens collected from children under 18 years of age [36]. In consistence with their results, we found *blaPER-1* within two out of five isolates recovered from patients younger than 15. However, both were ESBL-negative lacking *blaGES-1*. Higher frequency of *blaPER-1* among our isolates, particularly in children, indicates the increasing dissemination of this gene. As opposed to earlier studies performed in Iran, which reported lower percentage of *blaGES-1* (24.4% and 0%) [11,37] among ESBL strains, our data showed higher levels of *blaGES-1*-harboring *P. aeruginosa*. Moreover, we detected 61.1% CTX-M-positive isolates that were noticeably higher than reports from Shahcheraghi *et al*. [11] and Saeidi *et al.* [16]. Increased incidence of *blaGES-1* and CTX-M harboring isolates is a warning alarm for the health care system in Iran and is a potential situation for the emerging outbreaks in the future.

In the current study, the presence of *blaVEB-1, blaOXA-1, blaOXA-4* and *blaOXA-10* genes was identified in 20.4%, 23.1%, 47.2%, 7.4% of the isolates that is lower than previous reports from Iran. In Golshani and Sharifzadeh study [38], 64% out of 110 *P. aeruginosa* isolated from clinical specimens possessed the *blaOXA-10* gene. In that study, the MDR property was found in 36% of isolates [38]. Furthermore, the prevalence of OXA-10 and VEB-1 genes in the study done by Jabalameli *et al.,* was reported as 70% and 31.1% of the isolates, respectively. Their isolates were collected from burns [4]. In another Iranian study done on ESBL-producing isolates from burn-injured patients, the outbreak of *blaOXA-10 and bla*VEB-1 (74.62% and 31.34%, respectively) was higher than in our study [7]. It seems that the higher frequency of *blaOXA-10* among previous studies was due to a difference in the samples collection, which were isolated from burns. As opposed to several studies performed in other laboratories, which reported a higher percentage of *blaOXA-10* in Turkey (17.33%)[9], Thailand (94.44%) [39], Palestine (40.8%) [1], Egypt (41.7% of imipenem resistant isolates) [40] and in Korea (13.1% *blaOXA-10* and 0% *bla*VEB-1) [41], our data showed lower levels of *blaOXA-10*-harboring *P. aeruginosa.* However, the *bla*VEB-1 prevalence was higher than the findings from Egypt (10.4%) [40] and Korea (0%) [41]. In agreement with findings from Fazeli and his collaborators who found a lack of *blaOXA-10* among samples collected from children under 18 years of age [36], in this study, there is no *blaOXA-10* -harboring isolates among patients under 18.

In addition, we detected 47.2% *blaOXA-4*-positive isolates that were noticeably higher than the reports from Lee *et al*. (4.3%) (41), and Shahcheraghi *et al*. (17.1%) [37] .

Furthermore, we observed *blaOXA-1* in 23.1% of the isolates, which differed from reports from Iraq (100%) [5], two previous studies in Tehran, Iran (70.7%) [37] and Fazili *et al*., (0%) [36], but nearly agreed with a report from Palestine (22.5%) [1].

Overall, results from the current study demonstrated the growing emergence of resistant isolates in Iran. Access to updated data regarding antibiotic susceptibility and genetic background of *P. aeruginosa* is necessary to improve the healthcare system and attenuate the mortality and morbidity rate. Moreover, this information can be helpful for clinicians to prescribe appropriate antibiotics.

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FREQUENCY OF METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* STRAINS IN HEALTHCARE ASSOCIATED INFECTIONS IN THE REPUBLIC OF MOLDOVA

Greta Bălan[1], Serghei Covanțev[1*], Angela Cazacu-Stratu[2], Emilia Timbalari[1], Natalia Florea[1], Anatol Timbalari[1], Serghei Cebanu[2]

[1]Nicolae Testemițanu State University of Medicine and Pharmacy, Department of Microbiology, Chişinău, Republic of Moldova

[2]Nicolae Testemițanu State University of Medicine and Pharmacy, Department of Hygiene, Chişinău, Republic of Moldova

ABSTRACT

Staphylococcus aureus is a microorganism with an increased nosocomial potential that can be found worldwide. The aims of this study were: evaluating the frequency of isolation of methicillin-resistant *S. aureus* (MRSA) strains from various biological materials; establishing the ratio between MRSA strains and methicillin-susceptible *S. aureus* (MSSA) and MRSA strains, isolation frequency trend in various clinical departments; study MRSA strains resistance to non-β-lactam antibiotics.

The *S. aureus* strains were isolated from blood cultures, cerebrospinal fluid, pleural fluid, joint fluid, pus from surgical wound infections, abscesses, products from the lower respiratory tract (sputum, tracheal aspirate, bronchial aspirate). A total number of 1213 strains were isolated during the survey period from which 941 strains were included in the study. The proportion of MRSA strains was 40.2% (n = 378), with 39.89% (n = 165) in 2015 and 40.3% (n = 213); however, in 2016, the increase was not statistically significant (p>0.05).

Among the MRSA strains isolated during 2015, 45.8% were multidrug resistant, the percentage increasing to 79.5% in 2016 (p<0.001). The prevalence of MRSA strains varied depending on the hospital units profile and the clinical specimens. An increasing trend was observed in the frequency of MRSA isolation in patients of the intensive care unit. There were no strains exhibiting resistance to glycopeptidic antibiotics and linezolid.

REZUMAT

Staphylococcus aureus este un microorganism cu potenţial nosocomial crescut, care poate fi izolat în întreaga lume. Scopul studiului a fost evaluarea frecvenţei izolării tulpinilor de *S. aureus* rezistente la meticilină (MRSA) din diverse material biologice; stabilirea raportului dintre tulpinile MRSA şi *S. aureus* sensibile la meticilină (MSSA), tendinta şi frecvenţa izolării tulpinlor MRSA in diferite departamente clinice; evaluarea rezistenţei MRSA la antibioticele non-β-lactamice.

S. aureus au fost izolate din culturi de sânge, lichid cefalorahidian, lichid pleural, lichid articular, puroi din infecţiile chirurgicale, abcese, produse din tractul respirator inferior (spută, aspirat traheal, aspirat bronşic). Au fost izolate 1213 tulpini de *S. aureus* din care 941 tulpini au fost incluse in studiu. Proporţia de tulpini MRSA a fost 40,2% (n = 378), astfel, 39,89% (n = 165) în 2015 şi 40,3% (n = 213) în 2016, creşterea nu a fost semnificativă statistic (p> 0,05).

Printre tulpinile MRSA izolate în anul 2015, 45,8% au fost rezistente la mai multe medicamente, procentul a crescut până la 79,5% în 2016 (p <0,001). Prevalenţa tulpinilor MRSA a variat în funcţie de departamentul de profil şi material biologic. Creşterea incidenţei tulpinilor MRSA a fost relevant la pacienţii din secţia de terapie intensivă. Nu au fost identificate tulpini rezistente la glicopeptide şi la linezolid.

Keywords: healthcare associated infections, MRSA, MSSA, antibiotic sensibility.

INTRODUCTION

Staphylococcus aureus is a microorganism with an increased nosocomial potential that can be isolated worldwide. According to data released by NNIS (National Nosocomial Infection Surveillance), it is the most common cause of ventilation-associated pneumonia, surgical wound infections and catheter-related bacteremia [1]. In Europe the prevalence of healthcare-associated infections caused by methicillin-resistant *S. aureus* (MRSA) strains varies greatly, depending on geographic areas [2, 3]. MRSA strains percentage also shows large variations. According to the report of EARSS (European Antibiotic Resistance Surveillance System) in 2014, the percentage of MRSA strains isolated from blood cultures ranged from 0.9% (Netherlands) to 56.0% (Romania) [4]. The lowest values were determined in the Scandinavian

**Corresponding author: S*erghei Covanțev, Nicolae Testemițanu State University of Medicine and Pharmacy, Department of Microbiology, Address: 165, Stefan cel Mare si Sfant, Bd. MD-2004, Chişinău, Republic of Moldova, Phone +373 69948085, email: kovantsev.s.d@gmail.com

countries, the Netherlands, countries with a tradition of implementing strict measures to control healthcare-associated infections and the highest values were reported from countries in southern Europe.

Control of healthcare associated infections caused by MRSA involves the application of a special protocol. In order to implement it, first it is important to assess the local epidemiological situation, to improve the knowledge and characterization of circulating strains.

Therefore we evaluated the frequency of isolation of MRSA strains of various pathological products processed in the microbiological laboratory of the Republican Clinical Hospital (RCH) Chişinau, Republic of Moldova, during the years 2015-2016; established the ratio between MRSA strains and methicillin-sensitive *S. aureus* (MSSA), isolation frequency trend in various clinical departments; MRSA strains resistance to non-b-lactam antibiotics.

MATERIAL AND METHODS

Patients

The study was conducted from January 2015 until December 2016 by analyzing the data from the Microbiological Laboratory registers of RCH. The *S. aureus* strains were isolates from blood cultures, cerebrospinal fluid, pleural fluid, joint fluid, pus from surgical wound infections, abscesses, products from the lower respiratory tract (sputum, tracheal aspirate, bronchial aspirate). The samples obtained from ambulatory patients and the superficial products with mixed microbiota with over 3 species were excluded. We analyzed a single sample from every patient.

Bacteriological Studies

Identification of *S. aureus* species was performed by conventional bacteriological methods. Methicillin-resistance was tested using diffusion method as recommended by EUCAST (European Committee on Antimicrobial Susceptibility Testing) [5]. Confirmation of methicillin-resistance was performed by determining the minimum inhibitory concentration (MIC) of oxacillin standardized by microdilution method as recommended by EUCAST or E-test (EZY MIC™ Strips, HiMedia).

Antibiotic susceptibility assay

We determined the susceptibility / resistance profiles of MRSA strains to the following antibiotics: vancomycin (30 mg), erythromycin (15 mg), clindamycin (2 mg), gentamicin (10 mg), ciprofloxacin (5 mg), linezolid (30mg), tetracycline (30 mg), chloramphenicol (30mg) and trimethoprim - sulfamethoxazole (1.25 / 23,75 mg). MLS_B resistance phenotypes (macrolides, lincosamides, streptogramin B) were determined using the D test (double disc diffusion). Discs from HiMmedia (India) were used by the laboratory. Inoculum was prepared by making a direct saline suspension of isolated colonies selected from an 18- 24h blood agar plate. Turbidity of the suspension was adjusted to achieve equivalent to a 0.5 McFarland standard and five discs were applied on a 100 mm Mueller Hinton agar plate as per CLSI guidelines. Quality control strains were used: *S. aureus* ATCC 25923 (control disc diffusion sensitivity testing), *S. aureus* ATCC 29213 (control determination of minimum inhibitory concentration).

Statistical Analyses

Statistical analysis of data was performed using the program EpiInfo 2000. A $p < 0.05$ was considered to indicate significance.

RESULTS

Based on the criteria mentioned above, 1213 *S. aureus* strains were isolated from patients admitted during the survey period, from which 941 strains were included in the study. Of these, the proportion of MRSA strains was 40.2% (n = 378), thus 39.89% (n = 165) in 2015 and 40.3% (n = 213) in 2016, the increase being not statistically significant ($p>0.05$).

MRSA percentage varied depending on the pathological product (Table 1) and the hospital unit (Fig. 1).

The percentage of all MRSA strains of *S. aureus* strains was different depending on the department (Fig. 1).

Although during the survey period we did not observe a significant increase of MRSA frequency, MRSA isolation and distribution frequency trend evolved differently in the two years in different hospital units. In Fig. 2, the high risk departments for MRSA infections, respectively the intensive care and surgical units are shown.

Over the analyzed period of 2015-2016, we did not isolate MRSA strains resistant to vancomycin and linezolid. Resistance to non-b-lactam antibiotics is shown in Table 2.

Among MRSA strains isolated in 2015, 45.8% were multidrug resistant, the percentage increasing to 79.5% in 2016 ($p<0.001$).

Determination of non beta-lactam antibiotic resistance profiles of 198 MRSA isolates from 2016 revealed twenty four different resistance profiles,

Table 1 **- Specimen-wise distribution of *Staphylococcus aureus***

Specimen	2015			2016		
	Total	MRSA		Total	MRSA	
		n	(%)		n	(%)
Pus	321	121	37.7	339	98	28.9
Respiratory samples	64	26	40.6	127	89	70.1
Blood	17	11	64.7	27	20	74.1
Other specimens	11	7	63.6	35	7	20.0
Total specimens	413	165	39.89	528	213	40.3

MRSA – methicillin resistant *S. aureus*

Table 2 **- Resistance to non-b-lactam antibiotics based on strains of *Staphylococcus aureus* MRSA and MSSA isolates during the years 2015-2016**

Antibiotics	Strain (N)	Sensitive (%)	Resistant (%)	*p* value
Erythromycin	MRSA (365)	128 (35.1)	237 (64.9)	<0.001
	MSSA (828)	655 (79.1)	173 (20.9)	<0.001
Gentamicin	MRSA (348)	113 (32.5)	235 (67.5)	<0.001
	MSSA (796)	597 (75.0)	199 (25.0)	<0.001
Clindamycin	MRSA (362)	264 (72.9)	98 (27.1)	<0.001
	MSSA (827)	776 (93.8)	51 (6.2)	<0.001
Tetracycline	MRSA (362)	73 (20.2)	289 (79.8)	<0.001
	MSSA (825)	519 (62.9)	306 (37.1)	<0.001
Fluoroquinolone	MRSA (356)	128 (35.9)	228 (64.1)	<0.001
	MSSA (816)	564 (69.1)	252 (30.9)	<0.001
Chloramphenicol	MRSA (364)	310 (85.2)	54 (14.8)	<0.001
	MSSA (824)	785 (95.3)	39 (4.7)	<0.001
Trimethoprim Sulfamethoxazole	MRSA (364)	314 (86.3)	50 (13.7)	<0.001
	MSSA (823)	786 (95.5)	37 (4.5)	<0.001

MRSA – methicillin resistant *S. aureus*; MSSA - methicillin sensitive *S. aureus* ($p < 0.005$ is significant)

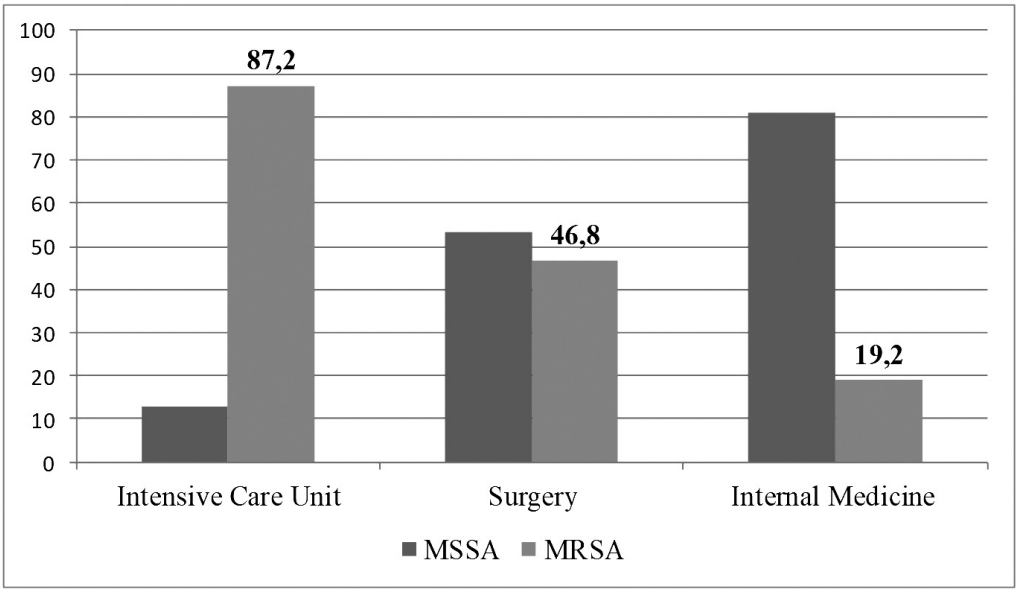

Fig. 1. Percentage of MRSA strains of *S. aureus* isolates depending on the type of the department

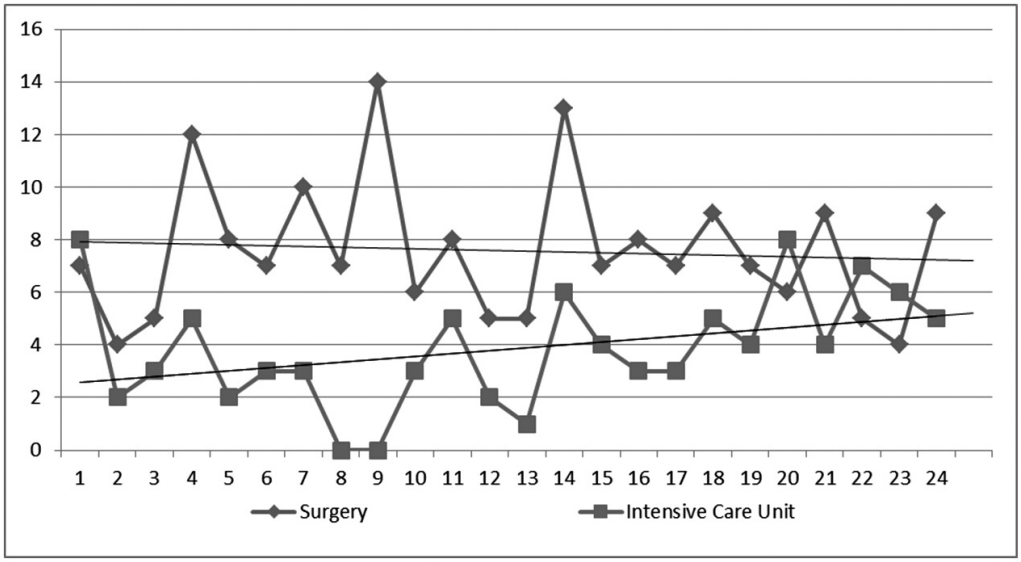

Fig. 2. Distribution and trend in MRSA strains isolation frequency surgical departments and intensive care

described based on the phenotypes of resistance to aminoglycosides (A), macrolides (M), lincosamides (L), tetracycline (T), fluoroqinolone (F), chloramphenicol (C), and trimethoprim-sulfamethoxazole (S). The most common antibiotic resistance profile was AMTF (n = 68), followed by AMLTF (n = 29), and ATF (n = 26). In the intensive care unit only 3 out of 24 resistance phenotypes were encountered: AMLTF profiles (n = 23), AMTF (n = 31) and ATF (n = 15). In surgical departments 18 profiles were detected whereas in internal medicine departments only 9.

Of the 198 MRSA strains, 5 were sensitive to all non-b-lactam antibiotics tested and only 7 exhibited resistance to one class of non-b-lactam antibiotics.

The MLS_B resistance phenotype was present in 184 MRSA strains. Of these, 146 exhibited MS (n = 77), inducible MLS_B (n = 63) and the constitutive MLS_B (n = 6).

DISCUSSION

MRSA in healthcare-associated infections loads hospital budget by increasing the cost of treatment and by prolonging hospitalization. MRSA spreads easily by people, who are colonized or infected, to patients. Most commonly, the spread is via the hands of health professionals. Firstly, the colonization of patients takes place, which is often favored by the use of broad spectrum antibiotics inactive to MRSA (cephalosporins, carbapenems, fluoroquinolones). Compared with infections caused by MSSA strains, healthcare-associated infections due to MRSA are associated with increased mortality [6]. MRSA does not have increased virulence as compared to MSSA, but because of its resistance to antibiotics commonly initiated empiric therapy is ineffective, having a negative impact on the development of infection [7].

The correct assessment of the phenomenon of resistance, a report MRSA/MSSA, it is important to study the strains isolated from relevant pathological specimens [8]. Therefore, we excluded from the study isolates [2] of superficial lesions that are frequently colonized with MRSA, without clinical significance. We also excluded MRSA from pharyngeal or nasal secretions since these regions are often colonized thus do not reflect infection and products with mixed bacterial more than three species, which usually reflects the poor quality of the pathological product and the clear role of MRSA cannot be established [9]. In the case of products from the lower airways, it is difficult to make the difference between colonizing and infecting agents. In this study we included MRSA isolates from bronchial aspirates, sputum or tracheal, if it was the only isolated strain or the predominant one.

We studied 941 strains of *S. aureus*, with MRSA strains percentage of 40.2%. The SENTRY study, which followed the epidemiology of MRSA in 25 university hospitals in Europe, reported a 25%, with variation between 2% (Utrecht, Netherlands) to 58% (Rome, Italy) [10].

If we look at the percentage of MRSA, based on the pathological product from which it has been isolated, it is noted that the largest share of MRSA

occurs when products are from the lower respiratory tract during 2016 (70.8%), showing a statistically significant increase compared to 2015 (40.6%). In the SENTRY study the highest percentage of MRSA was also isolated from products of the lower respiratory tract (34.4%) [10].

In the study we noticed an increase, although not statistically significant, of the share of MRSA in blood cultures (from 64.7% in 2015 to 66.6% in 2016). It is still debatable whether the relevance of these data requires further study to clarify the causes. It may be due to the low collection rate of blood cultures in RCH, which can lead to the overestimation of resistance. On the other hand, most of the positive blood cultures with *S. aureus* were from patients that were on antimicrobial therapy, which may be the consequence of the detection of resistant strains with overestimation of the predominant phenomenon of resistance.

There was a slight decrease in the number of MRSA isolated from the pus from 37.7% to 28.9% in 2015 to 2016, but without statistical significance ($p = 0.20$). The results can also be influenced by the conditions of collection of pus from the infected wounds. In 2015, a study in Chişinău reported 58.9% of MSSA strains isolated from infected wounds [11].

The report MRSA/MSSA during the survey period varies depending on the department and sections from which the bacteria were isolated. The high-risk department for MRSA infections was the intensive care unit, where strains of *S. aureus* were methicillin-resistant in 87.2% of cases. University hospitals in Europe that participated in the SENTRY study also demonstrate an increased rate of MRSA in intensive care units, but only 38% [10]. Instead, the EPIC study, which evaluated the percentage of MRSA in 1417 intensive care units from 17 Western European countries, reported a 60% of resistance [3].

During the study, there was no significant increase in the rate of MRSA. However, there is a tendency for an increase in MRSA strains isolation from the intensive care units and a small decrease in surgical departments.

During the study, there were no MRSA strains isolated with linezolid or glycopeptides resistance. It is not known whether among isolates were MRSA strains with reduced susceptibility to glycopeptides and strains with heteroresistance because diffusion method is not suitable for detecting these phenotypes.

Heteroresistant strains were reported for the first time in Japan in 1996. Since then many countries have reported the presence of strains with reduced susceptibility to vancomycin (VISA): Japan, France, United States (until 2002 - 8 strains confirmed) and Germany with isolates from MRSA infections. Heteroresistant strains can be encountered in Spain, Scotland, Hong Kong, Germany, Greece [12, 13]. Although these phenotypes are rare, it is necessary to introduce appropriate methods for their detection (brain-heart infusion agar supplemented with 6mg/mL vancomycin). In 2002 have been reported the first two strains of vancomicin-resistant *S. aureus* (VRSA) confirmed in the United States [14, 15], this phenotype is extremely rare.

In our study of methicillin-resistance was often accompanied by resistance to tetracyclines, macrolides, fluoroquinolones and gentamycin.

The presence of resistance to MLS_B group phenotypes was also studied (macrolide, lincosamide, streptogramin B). MS phenotype was detected in 77 (41.8%) of 184 MRSA strains tested, which is most commonly MLS_B phenotype. MLS_B constitutive phenotype was detected in 6 strains (3.3%). According to the literature, constitutive phenotype is associated mainly with methicillin-resistance and is the most common form of resistance in MRSA strains. In our study it was the least common phenotype. Inducible MLS_B phenotype was recorded in 63 strains (34.2%). According to the literature, this phenotype especially characterizes methicillin-sensible strains [16].

Although the data on non-β-lactam antibiotics resistance reported by different authors varies, all authors underline an increase in multi-resistant MRSA strains isolated in hospitals. Our study also noted this fact, with an increase in the percentage of multiresistant strains from 45.8% in 2015 to 79.5% in 2016 ($p<0,001$). During 2016, we identified 24 resistance phenotypes with different frequency and distribution in the departments of RCH. From them three phenotypes (AMTF, AMLTF and ATF) were most frequently isolated, the remaining phenotypes being found sporadically. Of the 24 only three phenotypes of MRSA were found in the ICU. It should be noted that patients from ICU were previously treated in other wards and ICU patients were transferred to various wards, so these MRSA strains could thus rapidly spread from a section to another.

Our study revealed that MRSA strains are endemic in RCH and require the implementation of specific measures to control the spread. In hospitals with highly endemic MRSA the control process can be difficult. Necessary measures to stop transmission

(isolating colonized/infected patients, increased staff compliance to hand hygiene, detecting nasal carriers among staff and patients newly hospitalized, presenting risk factors for colonization with MRSA) and optimize antibiotic use in order to reduce the selective pressure exerted by antibiotics are urgently required.

CONCLUSIONS

The prevalence of MRSA strains varied depending on the hospital units and pathological products. An increasing trend was observed in the frequency of MRSA isolation in patients of the intensive care unit. The majority of strains were multidrug-resistant. There were no strains with resistance to glycopeptides and linezolid.

FINANCIAL DISCLOSURE

The authors have nothing to disclose.

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TARGETING THE VIRULENCE FACTORS OF *SERRATIA MARCESCENS* BY AMBROXOL

Hisham A. Abbas*, Wael A. H. Hegazy

Department of Microbiology and Immunology, Faculty of Pharmacy, Zagazig University- Zagazig- Egypt

ABSTRACT

Serratia marcescens is an opportunistic nosocomial pathogen. It is highly resistant to antibiotics and can cause infection by secretion of virulence factors such as protease, prodigiosin and production of biofilm in addition to its ability to swim and swarm. To overcome the problem of antibiotic resistance, quorum sensing inhibition is an attractive target of new therapies for microbial infections. Quorum sensing controls the production of *Serratia marcescens* virulence factors and biofilm formation in addition to motility.

The objective of this study is the investigation of the ability of ambroxol to inhibit virulence factors, biofilm and swimming and swarming motilities of *Serratia marcescens*.

The minimum inhibitory concentration of ambroxol was determined by the microbroth dilution method. The effects of sub-inhibitory concentration of ambroxol on virulence factors, motilities and biofilm formation were detected. At sub-inhibitory concentrations ambroxol showed significant anti-virulence activities in a dose-dependent manner. It could inhibit biofilm formation by 82.16% at a concentration of 0.9 mg/ml and 65.23% at 0.3 mg/ml. The production of prodigiosin pigment was reduced by 59.30% to 93.58%. The inhibition of protease activity ranged between 27.42% and 90.86%. Moreover, it reduced the swimming to the level of 18.24% to 73.65% and swarming to the level of 27.27% to 75.76%. This anti-virulence activity of ambroxol may be due to quorum sensing inhibition.

In conclusion, ambroxol may be an anti-virulence agent for the treatment of *Serratia marcescens* infections.

REZUMAT

Serratia marcescens este un agent patogen nosocomial oportunist. Este foarte rezistent la antibiotice și poate provoca infecție prin secreția unor factori de virulență, cum ar fi proteaza, prodigiosina și producția de biofilm, în plus față de motilitatea sa în medii lichide și pe medii solide. Pentru a depăși problema rezistenței la antibiotice, inhibarea quorum sensing este o țintă atractivă a noilor terapii pentru infecțiile microbiene. Quorum sensing controlează producția de factori de virulență la *Serratia marcescens* și formarea biofilmelor în plus față de motilitate.

Obiectivul acestui studiu este investigarea capacității ambroxolului de a inhiba factorii de virulență, biofilmul și motilitatea la *Serratia marcescens.*

Concentrația minimă inhibitorie a ambroxolului a fost determinată prin metoda microdiluțiilor în mediu lichid. Au fost detectate efectele concentrației sub-inhibitoare a ambroxolului asupra factorilor de virulență, a motilităților și a formării biofilmelor. La concentrații sub-inhibitoare, ambroxolul a prezentat activități semnificative de antivirulență într-o manieră dependentă de doză. Aceasta ar putea inhiba formarea biofilmelor cu 82,16% la o concentrație de 0,9 mg/ml și de 65,23% la 0,3 mg/ml. Producția pigmentului prodigiosin a fost redusă cu 59,30% până la 93,58%. Inhibarea activității proteazelor a variat între 27,42% și 90,86%. Mai mult, a redus motilitatea în mediu lichid până la nivelul de 18,24% de la 73,65% și a scăzut pe mediu solid la nivelul de 27,27% de la 75,76%. Această activitate anti-virulență a ambroxolului se poate datora inhibării quorum sensing. În concluzie, ambroxolul poate fi un agent anti-virulență pentru tratamentul infecțiilor cu *Serratia marcescens.*

Keywords: *Serratia marcescens*, virulence factors, inhibition, ambroxol

INTRODUCTION

Serratia marcescens is an opportunistic Gram-negative human pathogen that can cause a variety of nosocomial infections such as urinary tract, respiratory tract and wound infections. This capability is enhanced by the production of virulence factors that can cause damage to human cells [1]. The treatment of infections caused by some strains of *Serratia marcescens* infections is problematic due to its high resistance to many antibiotics including β-lactam, aminoglycosides in addition to fluoroquinolone antibiotics [2, 3]. As a result, novel treatment strategies are necessary. One of these

**Corresponding author:* Hisham Abdel Monem Abdel Hamid Abbas, Address: Egypt, Zagazig, Zagazig University, Faculty of Pharmacy, Department of Microbiology and Immunology, e-mail: hishamabbas2008@gmail.com, Fax No.:(002)0552303266, Telephone No: (002)01276112647

therapeutic alternatives depends on targeting of the bacterial cell-to-cell communication system known as quorum sensing [4, 5]. *S. marcescens* utilizes the quorum sensing (QS) system to regulate the production of prodigiosin pigment, virulence factors such as proteases in addition to swimming and swarming motilities and biofilm formation [6, 7].

Ambroxol is a common mucolytic and expectorant that is used for patients suffering from asthma and chronic bronchitis [8]. It has antioxidant and anti-inflammatory activities [9]. Ambroxol was reported to prevent the adhesion and remove the attached cells of *P. aeruginosa* to cultured mammalian cells [10]. Due to its ability to block adhesion, quorum sensing and biofilm matrix production, ambroxol is a well-reported antibiofilm agent [11].

This study aimed to investigate the inhibitory activities of ambroxol as a quorum sensing inhibitor against virulence factors of *Serratia marcescens.*

MATERIALS AND METHODS

Media and Chemicals

Tryptone soya broth and Mueller Hinton broth were the products of Oxoid (Hampshire, UK). Luria-Bertani (LB) broth and LB agar were purchased from Lab M Limited (Lancashire, United Kingdom). Azocasein and ambroxol hydrochloride were purchased from Sigma (St. Louis, USA). Other chemicals were of pharmaceutical grade.

Bacterial Strains

One clinical *Serratia marcescens* isolate was obtained from endotracheal aspirate from an Intensive Care Unit patient admitted to Zagazig University Hospital. It was identified by MALDI-TOFF apparatus at the Clinical Pathology Department, Faculty of Medicine, Zagazig University.

Determination of Minimum Inhibitory Concentration (MIC)

To determine the minimum inhibitory concentration of ambroxol, the broth microdilution method was used according to the Clinical Laboratory and Standards Institute Guidelines (CLSI) [12]. Overnight cultures of the tested strains were prepared in tryptone soya broth (TSB). The cultures were diluted with Mueller-Hinton broth to have turbidities matching that 0.5 McFarland Standard. Further dilution with Mueller-Hinton broth (1:100) was performed so that approximate cell density of 10^6 CFU/ml was achieved. The bacterial suspensions were delivered in aliquots of 50 µl to the wells of a microtiter plate containing aliquots of 50 µl of different dilutions of ambroxol in Mueller-Hinton broth. The plates were incubated at 37°C for 20 hours and the MIC was calculated as the lowest concentration of the ambroxol that inhibited the visible growth in the wells.

Biofilm Formation and Inhibition Assay

The method of Stepanovic *et al.* [13] was used. Overnight culture of *Serratia marcescens* in tryptone soya broth (TSB) was diluted with tryptone soya broth to have an inoculum with 1×10^6 CFU/ml. The prepared suspension was added in aliquots of 100 µl to the wells of 96-well sterile microtiter plates with rounded bottom followed by incubation of the plates for 24 h at 25°C. In order to retain adherent cells only, the planktonic cells were removed by aspiration of the wells contents and washing of the wells three times with sterile phosphate buffered saline (PBS, pH 7.2). Methanol (99%) was used for fixation of biofilm cells for 20 minutes, after which it was removed and crystal violet (1%) was added for 20 minutes for biofilm staining. The unattached dye was washed off with distilled water and the plates were air-dried. The bound stain was dissolved in 33% glacial acetic and absorbance was measured with spectrofluorimeter (Biotek, USA) at 590 nm. The experiment was made in triplicates. To estimate the degree of biofilm production, the cut-off optical density (ODc) was calculated (3 standard deviations+ the mean OD of the negative control). The tested strain is non-biofilm producer (if OD ≤ ODc), weak biofilm producer (if OD > ODc, but ≤ 2x ODc), moderate biofilm producer (if OD>2x ODc, but ≤ 4x ODc), and strong biofilm producer (if OD> 4x ODc).

To test the biofilm inhibiting activity of ambroxol, the previous procedure was followed with and without sub-inhibitory concentrations of ambroxol (0.3-0.9 mg/ml).

The degree of biofilm inhibition was calculated using the following formula:

% of biofilm inhibition = (OD in absence of ambroxol-OD in presence of ambroxol) / OD in absence of ambroxol

Microscopic Analysis of Biofilm Inhibition

To visualize the inhibition of biofilm, 5 ml of the suspension prepared as previously mentioned [13] was added to polystyrene Petri plates in the absence and presence of 0.9 mg/ml of ambroxol. The plates were incubated for 24 h at 25°C and treated until they were stained with crystal violet (1%) for

20 minutes. The excess dye was washed off with distilled water and the plates were air-dried. Pieces of the plates were cut for microscopic examination. The biofilms were examined under the light microscope at a 400X magnification.

Swimming and Swarming Motilities Assay

The effect of ambroxol on the swimming and swarming motilities was investigated according to Matsuyama *et al*. [14] Swimming LB agar plates containing 0.3% (wt/vol) agar and swarming assay plates containing 0.5% (wt/vol) agar with and without sub-inhibitory concentrations of ambroxol were prepared. The plates were inoculated with 5µl of overnight culture in LB broth and incubated at 25°C for 20 h. The swimming and swarming diameters were measured. The experiment was made in triplicates and the results were averaged.

Prodigiosin Inhibition Assay

To assay for the inhibition of prodigiosin pigment in *S. marcescens* by ambroxol, overnight culture in LB broth was prepared and diluted 1% in fresh LB broth with and without ambroxol. After incubation at 28°C for 20 h, prodigiosin was extracted from the cells with 4% 1M HCl in ethanol. The concentration of prodigiosin was calculated by measuring the absorbance at 534 nm and the degree of inhibition was calculated. The experiment was made in triplicate and the results were averaged [15].

Protease Assay

Protease assay was performed by the azocasein assay method. Ambroxol-treated and untreated overnight cultures in LB broth incubated at 30°C were centrifuged at 8500 *g* for 15 minutes. One ml of azocasein (0.3%) in 0.05M Tris HCl and 0.5M $CaCl_2$ (pH 7.5) was added to 100 µl of the supernatant. After incubation for 15 minutes at 30°C, trichloroacetic acid (10%, 500 µl) was added to stop the reaction. The precipitated azocasein was removed by centrifugation and the absorbance of the supernatants was measured at 400 nm using Biotek Spectrofluorimeter (Biotek, USA) [16].

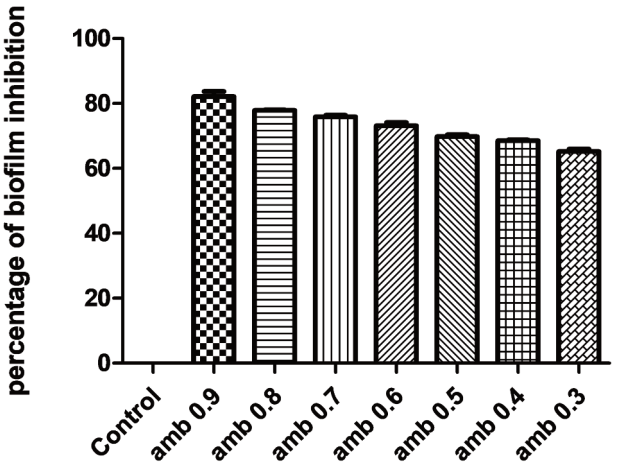

Fig. 1 – Inhibition of biofilm formation by sub-inhibitory concentrations of ambroxol. The error bars represent the standard errors of three measurements. Ambroxol inhibited biofilm formation in a dose-dependent manner

Statistical Analysis

The effects of ambroxol on virulence factors of *Serratia marcescens* were compared by One Way ANOVA followed by Dunnett's Multiple Comparison Test, Graph Pad Prism 5. *P* values <0.05 were considered statistically significant.

RESULTS

Determination of Antibacterial Activity

Ambroxol could inhibit the growth of the tested strain of *Serratia marcescens* at 3.75 mg/ml. The inhibitory effects against *Serratia marcescens* virulence factors were tested at concentrations equivalent to 1/4 MIC or less (0.3-0.9 mg/ml).

Assessment of Biofilm Formation and Inhibition

Serratia marcescens tested strain was strong biofilm forming because its OD was 1.139. This value was greater than 4 times ODc. The biofilm formation was tested in the presence of different sub-inhibitory concentrations of ambroxol and it was compared to the control in which no ambroxol was present. Ambroxol showed a significant concentration-dependent biofilm inhibiting activity ($P< 0.05$) (Fig. 1). At a concentration of 0.9 mg/ml, biofilm formation was inhibited by 82.16%, while at 0.3 mg/ml, the percentage of inhibition decreased to 65.23%.

Microscopic Analysis of Biofilm Inhibition

To demonstrate the effect of ambroxol on biofilm formation, microscopic examination of biofilms was performed in the absence and presence of 0.9 mg/ml of ambroxol using light microscope. Biofilm inhibition was observed in ambroxol-treated biofilms as compared to the biofilm in the control plate (Fig. 2).

Inhibition of Prodigiosin Production

Ambroxol showed a marked ability to interfere with quorum-sensing dependent production of prodigiosin pigment. The production of prodigiosin was significantly reduced by ambroxol in a dose-dependent manner as compared to the control ($P< 0.05$) (Fig. 3). Ambroxol reduced prodigiosin to the level of 59.30% to 93.58%.

Inhibition of Protease Production

In the azocasein assay, ambroxol (0.3-0.9 mg/ml) could diminish protease production ($P< 0.05$). Protease inhibition was highest at 0.9 mg/ml (90.86%) while 0.3 mg/ml showed the lowest inhibition activity (27.42%) (Fig. 4).

Inhibition of Swimming and Swarming Motilities

Swimming and swarming motility were significantly inhibited by ambroxol in a concentration-dependent manner ($P < 0.05$). The inhibition of both swimming and swarming was correlated. Ambroxol (0.9 mg/ml) could diminish swimming motility by 73.65%, while at 0.3 mg/ml the reduction of swimming was 18.24% (Fig. 5). Swarming was inhibited to the level of 75.76% at 0.9 mg/ml, but at 0.3 mg/ml, the inhibition level was only 27.27% (Fig. 6).

DISCUSSION

Quorum sensing inhibitors are compounds that interfere with the virulence-controlling quorum sensing system. They do not affect bacterial growth, and as a result, the emergence of resistant strains of bacteria could be avoided [17]. *Serratia marcescens* uses quorum sensing to regulate the expression of genes responsible for motility, biofilm production and prodigiosin in addition to secreted virulence factors [1]. Protease has a vital role in human infections [18]. Prodigiosin is a red pigment that has immunomodulatory effects [6]. Biofilms are communities of sessile organisms attached to a surface and housed within a matrix [19]. Biofilm-based infections are problematic to treat due to their extreme resistance to antibiotics and the host immunity [20, 21].

In this study, ambroxol showed antibacterial activity against *Serratia marcescens*. The growth was inhibited at 3.875 mg/ml. Sub-MICs of ambroxol (0.3-0.9 mg/ml) were used to investigate the effect on virulence factors production, biofilm formation and motilities of *Serratia marcescens.* Ambroxol showed significant dose-dependent inhibiting activities against prodigiosin, protease and

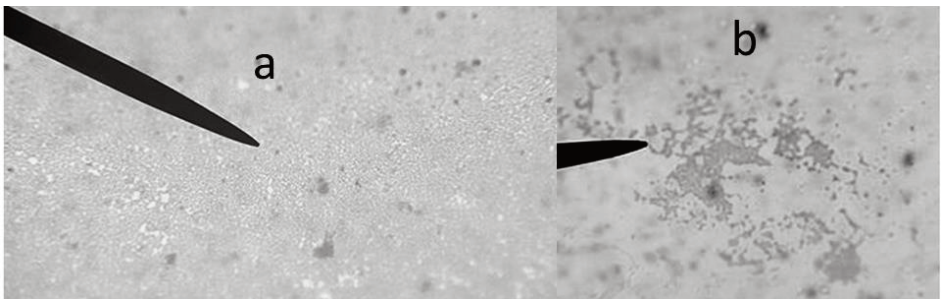

Fig. 2 – Microscopic visualization of biofilms.
Biofilm in the absence of 0.9 mg/ml of ambroxol (a).
Significant reduction in biofilm production in the presence of 0.9 mg/ml of ambroxol (b)

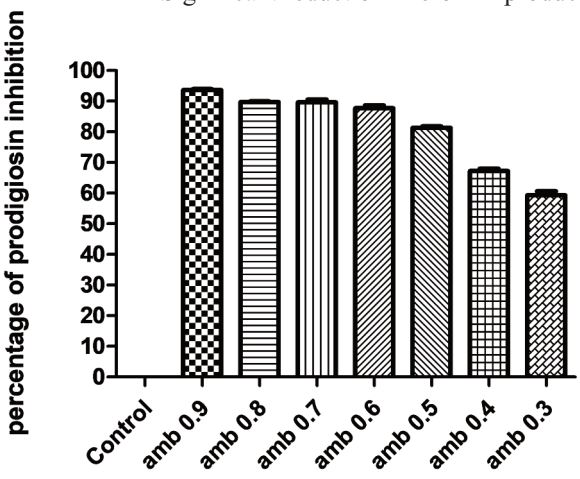

Fig. 3 – Inhibition of prodigiosin by sub-inhibitory concentrations of ambroxol. The error bars represent the standard errors of three measurements. Ambroxol inhibited prodigiosin production in a concentration-dependent manner.

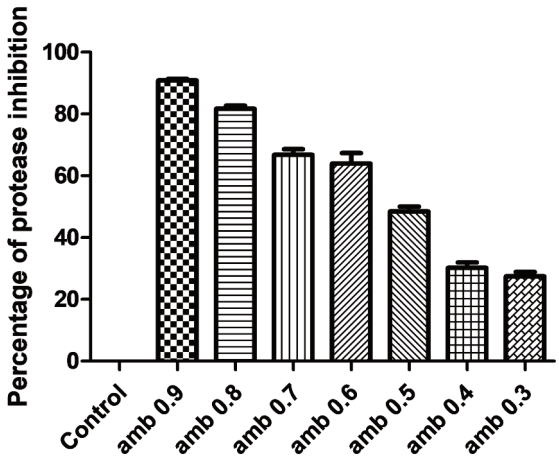

Fig. 4 - Inhibition of protease by sub-inhibitory concentrations of ambroxol. The error bars represent the standard errors of three measurements. Protease inhibition by ambroxol was concentration-dependent.

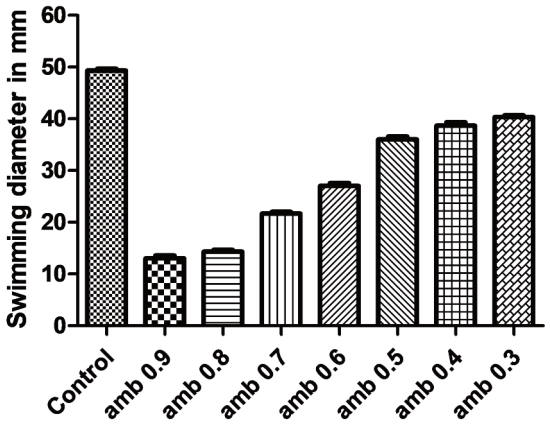

Fig. 5 – Inhibition of swimming motility by sub-inhibitory concentrations of ambroxol. The error bars represent the standard errors of three measurements. Ambroxol blocked swimming motility in a dose-dependent manner.

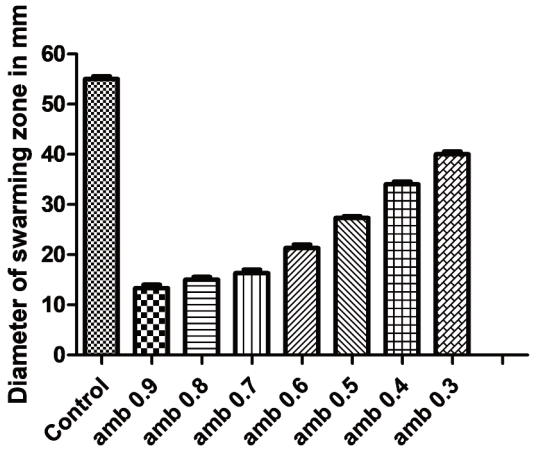

Fig. 6 - Inhibition of swarming motility by sub-inhibitory concentrations of ambroxol. The error bars represent the standard errors of three measurements. Swarming motility was reduced in a dose-dependent manner.

biofilm production in addition to its effect on inhibiting swimming and swarming motilities.

The activity of ambroxol against virulence factors of *Serratia marcescens* may be due to quorum sensing inhibition. Ambroxol was previously reported as a quorum sensing inhibitor in *Pseudomonas aeruginosa* at concentrations of 1.875-3.75 mg/ml [11]. Moreover, it has shown a blocking activity against swimming, swarming and biofilm formation in *Proteus mirabilis* at concentrations of 0.5-0.9 mg/ml [22]. The use of ambroxol has the advantage of being approved by the Food and Drug Administration (FDA). As a result, it can find its way to clinical application. This is very important because most quorum sensing inhibitors cannot be used clinically due to their high toxicity to humans [23].

CONCLUSION

Ambroxol may be a beneficial agent in the treatment of *Serratia marcescens* infections. This activity may be due to quorum sensing inhibition that controls the expression of virulence factors. The anti-virulence therapy is an alternative option to overcome the problem of antibiotic resistance.

Conflict of Interests: There is no conflict of interest

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REVIEW: CYTOTOXICITY AND GENOTOXICITY ASPECTS OF ZNO AND SILVER NANOPARTICLES DESIGNED FOR ANTIMICROBIAL APPLICATIONS

Carolina-Antonia Mărchidanu[1,2], Magdalena-Valentina Lungu[3], Irina Gheorghe[1,2], Mohammed Dyia Hussien[1,2], Ancuța Telcian[1,2], Grațiela Grădişteanu Pîrcălăbioru[2], Liliana Burlibaşa[1], Nicoleta Constantin[1], Mariana Carmen Chifiriuc[1,2*]

[1,2]University of Bucharest, Faculty of Biology, Microbiology Department, Bucharest, Romania
[1,2]Research Institute of the University of Bucharest – ICUB, Life, Environment and Earth Sciences Division, Bucharest, Romania
[3]National Institute for Research and Development in Electrical Engineering ICPE-CA, Advanced Materials Department, Bucharest, Romania

ABSTRACT

The emergence of nanotechnology has enabled a wide range of nanoparticles (NPs) to be developed for specific applications in the pharmaceutical, cosmetic and other biomedical industries, as well as for the development of imaging diagnosis techniques. The huge potential of NPs for different applications is due to their atomic scale size at which they are more reactive than conventional size particles. However, this property also increases the risk for the occurrence of a greater cytotoxicity. The purpose of this paper was to review the mechanisms of the antimicrobial activity of inorganic NPs based on silver and zinc oxide, as well as their cytotoxic and genotoxic effects.

REZUMAT

Apariţia nanotehnologiei a permis dezvoltarea unei game largi de nanoparticule (NP) pentru aplicaţii specifice în industria farmaceutică, cosmetică şi alte industrii biomedicale, precum şi pentru dezvoltarea tehnicilor de diagnosticare imagistică. Potenţialul imens al NP pentru diferite aplicaţii este rezultatul dimensiunii la scală atomică la care sunt mai reactive comparativ cu particulele de dimensiuni convenţionale. Această proprietate a NP favorizează de asemenea riscul apariţiei unei citotoxicităţi crescute. Scopul acestei lucrări a fost de a revizui mecanismele activităţii antimicrobiene a NP anorganice pe bază de oxid de zinc şi de argint, precum şi efectele lor citotoxice şi genotoxice.

Keywords: nanoparticles, silver, zinc oxide, cytotoxicity, genotoxicity, antimicrobial

INTRODUCTION

Over the last decade, there has been an exponential increase in the application of nanoscience and nanotechnology, which has led to significant advances in the development of new nanomaterials.

Nanotechnologies are becoming increasingly important in various areas, such as health, environmental protection, cosmetics, food, electronics, energy, and medicine [1].

This increase in innovation is largely due to the special properties these nanomaterials possess, leading to mechanical, dimensional, electrical, photochemical and catalytic growth [2].

For example, the multiple biological and medical applications of silver NPs have increased the risk for live organisms to be exposed directly or indirectly to them, which could induce many harmful effects on human health and the environment. It is well known that smaller metallic metal and metal oxide NPs induce a higher toxic potential towards cells due to their tendency to release metal ions in the physiological environment [3]. Side effects (primary or secondary) of silver NPs may extend into the cardiovascular system or central nervous system, thus causing neurotoxicity or immunotoxicity [4].

Despite the widespread use of zinc oxide (ZnO) NPs, the safety of this compound for humans is still unclear [5].

Although ZnO is presented as "generally recognized as safe (GRAS)" by the Food and Drug Administration and also used as a food additive, it is also suggested that exposure to ZnO NPs could lead to a genotoxic potential mediated by lipid peroxidation and oxidative stress.

Therefore, there are still many unsolved issues regarding the safety and the latent risks to human health and to the environment [6].

In this context, the purpose of this paper was to review the mechanisms of the antimicrobial activity of inorganic NPs based on silver and ZnO, as well as their cytotoxic and genotoxic effects.

*Corresponding author: Mariana Carmen Chifiriuc, University of Bucharest, Faculty of Biology, Microbiology Department, Bucharest, Romania, email: carmen.chifiriuc@gmail.com

1.1. Methods used for the synthesis and characterization of Silver and ZnO NPs

Nanomaterials or nanostructures can be synthesized by a variety of techniques such as pulverized pyrolysis, thermal decomposition, molecular beam epitaxy, chemical vapor deposition, and laser ablation [7]. In general, NPs synthesis is performed using three different approaches: physical, chemical and biological [8].

Some of the most important physical approaches are laser ablation and evaporation-condensation processes, using a tubular oven at atmospheric pressure. The advantages of the physical methods compared to the chemical ones are: the absence of solvent or other dangerous chemicals and the uniformity of NPs [1].

Chemical synthesis is one of the most important techniques that uses a range of precursors and different conditions such as temperature, time, reagent concentration. The variation of these parameters leads to morphological differences in the size and geometry of the resulting NPs [7]. The chemical approach uses water or organic solvents to produce metal NPs. This process usually uses three main components: metal precursors, reducing agents and stabilizers. Basically, the reduction of metal (such as silver) salts involves two steps: the nuclear phase and a subsequent growth. The chemical methods involve the use of several techniques: cryochemical synthesis, laser ablation, electrochemical reduction, laser irradiation, thermal decomposition and chemical reduction. These methods have a high yield and low cost [8].

The biological approach can be used to synthesize different NPs without the use of expensive, harmful or toxic substances. Many studies have been conducted on the synthesis of silver NPs successfully using microorganisms and biological systems. For example, bioreductive synthesis of silver NPs has been demonstrated using *Fusarium oxysporum* [1].

The most commonly used NPs characterization methods are electronic transmission microscopy (TEM) for: size, morphology and agglomeration, dynamic dispersion of light (DLS) for particle size distribution, zeta potential measurement for nanoparticle surface loading, and x - Ray diffraction (XRD) for the crystalline particle structure. In some cases, such as gold and silver NPs, UV spectroscopy can be used to determine the size and size distribution due to size dependent optical activity. Analysis of NPs should be repeated after administration, as various modifications may occur during the application process. NPs are usually applied by mixing with the cell culture medium. The components dissolved in the medium, especially the ions, lead to the agglomeration and precipitation of many NPs, causing significant changes in their physicochemical properties. Similar effects are expected when NPs come in contact with surfactants or other biological fluids. It has been demonstrated that some NPs tend to form protein crowns in biological systems [9].

1.1.1. Silver NPs

Silver NPs are particles with sizes between 1 nm and 100 nm. Recent attention has been focused on silver NPs due to their distinct physicochemical and biological properties. Their multidisciplinary application is generally known, although their use as antimicrobial against a wide range of bacterial and fungal strains is the most studied [3-4, 10].

There are different synthesis methods for obtaining silver NP, for example, laser ablation, gamma irradiation, electronic irradiation, chemical reduction, photochemical methods, microwave processing and synthetic biology methods. As for silver precursors for the synthesis of these NPs, silver nitrate is most often used because it is cheap and affordable. A reducing agent must be applied to convert silver from the ionic form to the element [11]. The biological synthesis of this type of NPs from bacteria was achieved by mixing *Bacillus subtilis* culture supernatants using the microwave irradiation method [1].

Silver NPs can be also synthesized extracellularly using *Fusarium oxysporum* mold isolates without any evidence of particle flocculation even one month after the reaction. The long-term stability of the solution given by NPs may be due to the stabilization of silver NPs by proteins. The morphology of NPs varies with a generally spherical shape and occasionally triangular. Stable silver NPs can be obtained also using *Aspergillus flavus* or *A. fumigates* strains [1].

The *Camellia sinensis* extract was used as a reducing and stabilizing agent for biosynthesis of silver NPs in aqueous solutions and in ambient conditions. It was observed that when the amount of extract increased, the resulting NPs were relatively larger, wide and spherical. Biomolecules such as phenolic acids (eg caffeine, theophylline) present in the *C. sinensis* extract are responsible for the formation and stabilization of silver NPs. Extracts from the black tea leaves are also used in the

production of silver NPs, they are stable and have different shapes (spherical, trapezoidal, prisms, rods). Polyphenols and flavonoids are responsible for the biosynthesis of these NPs [1].

1.1.2. ZnO NPs

ZnO NPs have extraordinary physical properties. The ZnO semiconductor has some unique properties, such as good transparency, high electron mobility, a broadband gap, and strong luminescence [7]. It is important to note that the physical properties of the semiconductor materials at nanoscale undergo changes known as "the effects of the quantum dimension" [7].

The intrinsic optic properties of ZnO nanostructures are extensively studied for the implementation of photonic devices. It has been shown that the photoconductivity of ZnO nanowires is influenced by the desorption-adsorption process of O_2. At illumination, photogenerated electrons significantly increase conductivity, while when illumination is stopped, O_2 molecules reabsorb onto the surface of nanowires and reduce conductivity [7].

ZnO is a commercially available widely used material that has recently gained interest in researchers and nanotechnologies due to its considerable antimicrobial protection properties.

The steam transport process is the most common method for ZnO synthesis. In this process, Zn and oxygen vapors are transported and react with each other leading to the formation of ZnO nanostructures. There are many ways to generate zinc and oxygen vapors. ZnO decomposition is a lighter, more direct and simple method. However, it is limited to very high temperatures, such as ~ 1400˚C. Another direct method involves heating the zinc dust into the oxygen stream. This involves a relatively low rise of temperature (500-700°C), but the ratio of zinc vapor pressure to oxygen pressure must be carefully controlled to obtain the desired nanostructures. It has been observed that the change in this ratio results in a great variation in the morphology (size and geometry) of the nanostructures [7].

Hydrothermal technique is an efficient alternative synthesis method because of the decrease in process temperature, rendering much easier the control of the particle size. This process has several advantages, such as the use of simple equipment, catalyst-free growth, low cost, uniform production and less dangerous than other growth processes. This technique has been used successfully for the preparation of ZnO NPs and other luminescent materials. Morphology and particle size can be controlled by the hydrothermal process by adjusting the reaction temperature, time and precursor concentration [7].

The chemical synthesis of ZnO NPs uses mostly alchoolic media, such as ethanol, methanol or propanol. In alcoholic environments, the growth of oxide particles is slow and controllable [7]. In the precipitation process, ZnO can be synthesized by using zinc nitrate and urea as precursors. The biological synthesis of NPs using microorganisms can be difficult because it involves an elaborate process of cell culture maintenance, intracellular synthesis and multiple purification steps [7].

1.2. Practical applications of Silver and ZnO NPs

1.2.1. Silver NPs

Silver NPs have been shown to be widely deployed in the field of biomolecular detection, sensing, diagnosis, catalysis, transport and microelectronics [12]. It has been observed that silver NPs have a higher marketing value than other NPs, and their presence in consumer products is very popular. This remarkable popularity can be attributed to the antimicrobial properties of ionic silver. Among metallic NPs with special antimicrobial activity, those made of silver are extremely effective bactericides. The antibacterial activity of silver ions is frequently tested on Gram positive bacteria (*Staphylococcus aureus*) and Gram negative (*Escherichia coli*) isolates [13]. Antimicrobial activity of silver NPs is among the main reasons for their use in the formulation of surface detergents, toys, air and water disinfection, antimicrobial catheters, clinical clothing, and food preservation.

Silver NPs of various shapes and sizes have been used to develop a rapid diagnostic device for the detection of acute systemic fever, such as yellow fever and Ebola virus. It is also of great interest to include silver NPs in a wide range of medical devices, such as bone matrix, surgical instruments, surgical masks, wound dressings and antibacterial creams [10]. The use of silver NPs on implant surfaces is of considerable interest, particularly due to antimicrobial properties and strong anti-biofilm potential. Silver NPs effectively inhibit the bacteria growth including multidrug resistant strains at very low concentrations in mg/L units, while such concentrations do not show acute cytotoxic effects, as evidenced by concentrations higher than 20 mg/L [13].

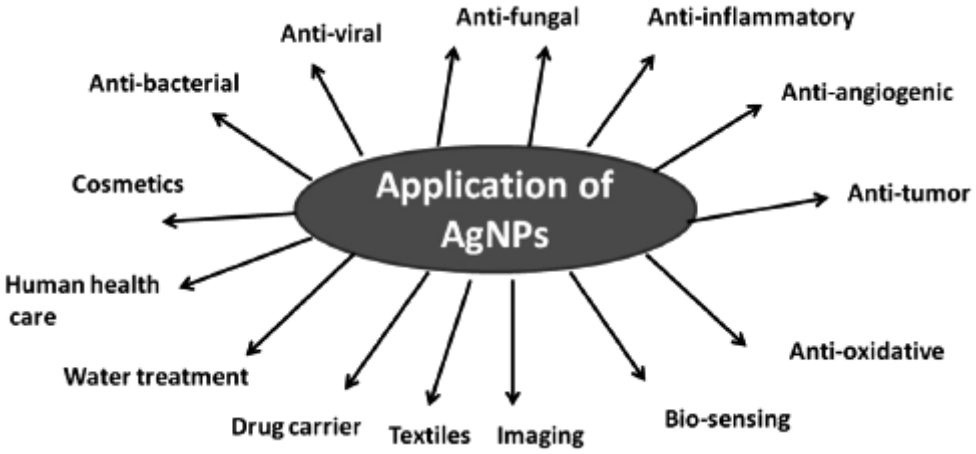

Fig. 1 - Biomedical applications of silver NPs (adapted after Zhang *et al.,* 2016)

Another main use of silver NPs is their incorporation into textile products, for the appropriate manufacture of cotton fabrics with antibacterial capabilities and superhydrophobic properties that can be used as protective clothing to work in wet and less healthy areas. In addition, silver NPs have been tested for many industrial applications, including disinfection of waste water treatment plants and silicone rubber gaskets for the preservation and transport of paper and textile materials. Given that silver has long been known to have a strong toxicity for a wide range of microorganisms, it is a great advantage to use silver compounds for antimicrobial applications against food-borne pathogens as well as antioxidants to maintain food quality [12].

Due to their unique antibacterial, antifungal, anti-cancer and antiangiogenic properties, silver NPs have been widely used in household tools, healthcare and food silverware, environmental and biomedical applications (Fig. 1) [8].

Considering intrinsic biocidal activity, silver based materials have been used for years as antimicrobials for the treatment of burns, trauma and diabetic ulcers. Different silver products have become effective alternatives to commercially available antibiotics [3]. Silver containing dressings can be successfully used to treat both acute wounds, such as surgical or traumatic (including burns) ones and chronic wounds, including localized open or closed lesions, by preventing microbial spread and subsequent systemic infection. Silver NPs are likely to have multiple antibacterial mechanisms, but the exact basis for silver NPs antimicrobial activity is still unknown.

Silver NPs penetrate the cell wall of Gram negative bacteria, leading to increased cell permeability, followed by cell death. The formation of free radicals by silver NPs is attributed to membrane damage.

Some studies have shown that silver NPs release silver + ions in the presence of water [13]. Silver atoms attach to the thiol groups of enzymes involved in vital biological processes, including ion transport and transmembrane energy generation. Silver catalyzes the oxidation reactions between oxygen and hydrogen atoms of thiol, producing disulfide bonds and inhibiting bacterial growth [14]. Changes in vital cell biological molecules lead to changes in the cellular structure that can lead to their death. The strong binding capacity of silver + with phosphorus-containing bases, such as DNA bases, results in the inhibition of bacterial growth and cell death [15].

Another mechanism of the antimicrobial activity of silver is the interruption of bacterial cellular respiration. Silver ions are able to disable ribosomal 30S subunits. Disabling the ribosome complex leads to interference with the proteins translation, an important step in bacterial multiplication [16].

The silver ions bind competitively with the metal chelating sites of different enzymes directly influencing various critical cellular functions, including cellular respiration and oxidation, cell wall integrity and cell transport / permeability.

In most cases, silver NPs have emerged as alternatives to antibiotic resistance problems because silver NPs use multivalent mechanisms to exhibit antibacterial activities. In addition, the large area-to-volume ratio and the specific physical and chemical characteristics of silver NPs make them effective against MDR bacteria, which include methicillin

resistant *S. aureus* (MRSA), ampicillin resistant *E. coli*, vancomycin resistant *S. aureus* (VRSA), erythromycin resistant *Streptococcus pyogenes*, MDR *Pseudomonas aeruginosa* isolates [15].

Key factors, such as size and shape, affect the antibacterial activity of silver NPs. The smaller the size of antimicrobial particles, the higher the surface-to-volume ratio. The large surface offers many opportunities for interaction with bacteria. The triangular silver NPs have been shown to be more active than spherical and rod-shaped silver NPs against *Escherichia coli*, suggesting that the shape of silver NPs is a key factor in determining antimicrobial activity [15].

In addition, for silver NPs, bacterial resistance has not been reported so far, despite the fact that ionic silver resistance has been observed. With a very low risk of developing bacterial resistance, it is relevant to know the antibacterial effects of silver NPs. This is an extremely valuable effect, especially today, when we face increased antibacterial resistance to antibiotics and other antibacterial substances. Therefore, a multidisciplinary joint action, including antibiotic replacement with non-antibiotic approaches, could reduce the bacterial resistance rate [17].

Bacterial biofilms are a serious problem due to the increasing ineffectiveness of conventional antibiotics, thus numerous alternative methods of controlling bacterial biofilms are considered. Silver NPs have recently received increased attention for their antimicrobial effects and possible clinical applications.

1.2.2. ZnO NPs

ZnO NPs have attracted intense research efforts for their unique properties and versatile applications in electronics, ultraviolet (UV), piezoelectric, chemical and electronic centrifugal sensors. The high catalytic activity of ZnO NPs makes them an important industrial additive for many products including plastics, cement, glass, rubber, lubricants and food, and their excellent absorption and UVA and UVB reflection have also made them a common component in cosmetics and sunscreens [18]. ZnO NP powders are widely used in other cosmetic products (foot care, ointments and over-the-counter products), pigments and coatings (ultraviolet, fungicide in paints) catalysts. Because of their targeting potential, ZnO NPs could be used to treat cancer and / or autoimmunity [7]. ZnO NPs can be also used for the photocatalytic degradation of environmental pollutants [7]. ZnO NPs have shown beneficial effects on bacteria such as *Pseudomonas putida*, which has the potential for bioremediation and is a strong colonizer of plant roots [7].

Moreover, the use of ZnO NPs has expanded to consumers in the form of textiles as well as in the electronic products industry [19]. ZnO NPs have antimicrobial properties (antibacterial and anti-fungal), making them useful for biomedical applications and food package and preservation. They can be applied as a powerful sanitary agent for disinfecting and sterilizing food industry equipment and containers against attack and contamination with food-borne pathogens.

A large number of commercial materials have used ZnO as an active component, such as bandages, socks, dressings [20]. ZnO NPs revealed excellent *in vivo* results in dermatological applications such as sunscreens and cosmetics, while they have limited penetration into the skin layers and little negligeable on skin cell activity (e.g. morphology, metabolism or oxidation) [20] .

ZnO NPs are known to be effective against several types of bacteria and fungi, both under ambient light and in the absence of ultraviolet (UV) light. Different morphologies of ZnO NPs have been studied in order to elucidate the mechanisms underlying their antimicrobial effects, and although the precise mechanism remains unclear, several theories have been proposed, including the generation of reactive oxygen species (ROS) or release of Zn^{2+} ions. Studies have also shown that the antibacterial activity of ZnO NP against *E. coli* and *Candida albicans* isolates may be due to hydroxyl radicals generated by interactions between ZnO NPs and water. Another mechanism involves the direct interaction between ZnO NPs and cell surfaces that affects cell membrane permeability; afterwards, these NPs could penetrate into bacterial cells and induce the oxidative stress, which leads to inhibition of cell growth and ultimately to cellular death. Gram positive bacteria, such as *S. aureus* isolates are more sensitive to ZnO NPs than Gram negative ones, such as *E. coli* strains [18].

Although ZnO NPs may play a beneficial role when used against pathogenic microorganisms, they can adversely affect environmental bacteria and could be capable of altering the ecological balance in soil environments [18].

It has been observed that the antimicrobial activity of ZnO NPs synthesized by biological or chemical pathways is similar. In exchange, an increased bioactivity was attributed to smaller particles due to their high surface / volume ratio [7].

1.3. Toxicity of silver and ZnO NPs

Accumulated evidence clearly shows that exposure to NPs may be toxic to the biological system, from prokaryotes to higher eukaryotes, including humans. Despite their antimicrobial properties, silver and ZnO NPs exhibit cytotoxicity and genotoxicity to animals and humans. Previous studies have shown that both the cytotoxic and genotoxic effects of these NPs, as well as their interaction with cells, depend on both size and chemical coverage. Exposure to NPs may occur by dermal contact, oral administration, inhalation, contact with mucous membranes and / or blood circulation. It has also been demonstrated that response to toxicity varies depending on cell type or tissue. Furthermore, the mechanism of toxicity or cell damage may be different in different cell types, suggesting that the selected cell may have a profound impact on the outcome of a particular study [21]. Previous studies have argued that smaller particles can cause more toxicity than larger ones because of their larger surface area [8]. Several studies have shown that exposure to NPs leads to an inflammatory response, DNA damage, oxidative stress, lipid peroxidation, apoptosis, micronucleus formation, gene expression modification, genotoxicity, cytotoxicity, reproductive toxicity, immunotoxicity and non-genotoxic carcinogenicity. Different mechanisms have been proposed for non-genotoxic carcinogenicity, one of which is the epigenetic change of the DNA methylation model which may lead to modified gene expression.

Most of the current nanotoxicological research uses *in vitro* models that do not provide information about NP activity in host organisms (biodistribution, accumulation, metabolism, persistence, elimination, etc.) [6]. In addition, the protocols used in various nanotoxicity studies are not standardized with regard to the numerous variables in this area of research (variations in size, manufacturing procedures, aggregation, solubility, intracellular absorption and cell and animal models). These aspects prevent any comparison or reproducibility of the results obtained, raising the need for standardization and the establishment of experimental models *in vitro* / *in vivo* for the characterization of cytotoxicity and NP biocompatibility. It is necessary to establish standard pharmacological parameters such as the dose, the administration route, metabolism etc. [6].

Several model organisms such as *Drosophila melanogaster*, rainbow trout (*Oncorhynchus mykiss*), nematode (*Caenorhabditis elegans*), algae, daphnia, mice, rats etc. have been used to understand the molecular mechanisms of NPs cytotoxicity and genotoxicity [22]. Global acceptance of zebrafish (*Danio rerio*) as a modern experimental animal model is gradually increasing due to an exceptional set of features they possess. Some of these are their small size, very high reproducibility, rapid development, embryo transparency and bias on genetic and chemical screens. In addition, we can find extensive literature on experiments on zebrafish. It has been observed that zebrafish is a small animal and can therefore be handled without difficulty. Eggs are cut off quickly and larvae can begin feeding after 120 hours of fertilization, indicating the onset of experiments on the larvae over zebra from that point. Another advantage is that organs and tissues can be readily visualized *in vivo* and can be instantly examined. In addition, it has also been noticed that eggs degrade rapidly and organogenesis occurs rapidly. As a result, major organs develop in 5-6 days after fertilization in larvae.

Females can reach a size of 38 mm, while males can reach a maximum average size of 35 mm with a weight of 0.9 and 0.6 g, respectively. Another major advantage of the zebrafish is that the cardiovascular, nerve and digestive traits of this animal model are similar to mammals. In addition, highly conserved signaling pathways are found both in zebra and in humans with a high level of genomic homology. The high level of similarity between human genome and zebra (more or less 75% similarity), is making it a feasible animal model for analytical studies [22]. Due to all these reasons, this animal model is becoming popular in the fields of toxicology and biomedical research during adult and embryonic stages.

However, there is still need to validate animal models for NPs toxicity studies. Difficulties consist in developing a fair approach to interpreting studies and making decisions on the parameters to be considered when examining toxicity *in vivo* systems [23].

The evaluation of NPs genotoxicity can be achieved by the micronuclei test, consisting in the evaluation of the frequency of micronucleated cells using mouse bone marrow or peripheral blood cells, after the administration by different routes (intraperitoneal injection, oral gavage), in different dosages and treatment schemes [24].

The *in vivo* analysis of rodent micronuclei has limitations as other genotoxicity tests, namely: no test can detect all genotoxic chemicals with different mechanisms of action. However, the *in vivo* micronucleus test was widely used, being the most

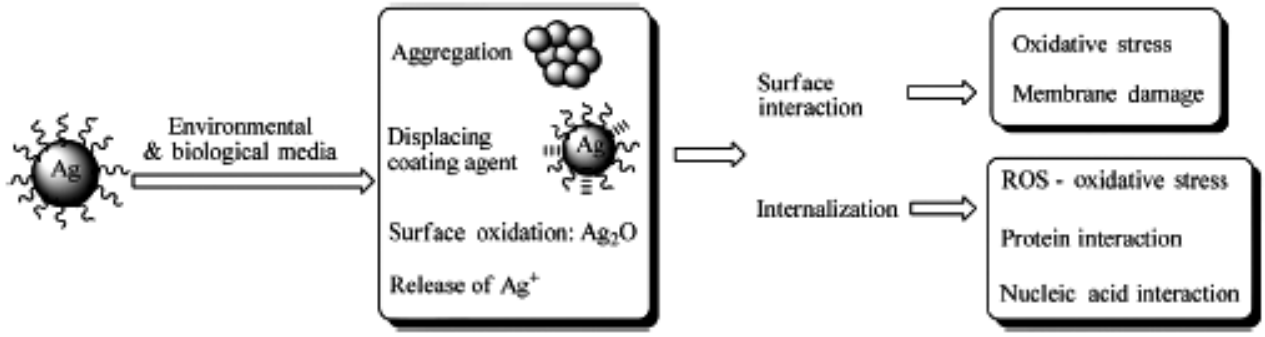

Fig. 2 - The toxicity of silver NPs in the biological environment (McShan *et al.,* 2014)

reliable test to assess the presence of chromosomal aberrations [24].

Micronucleus analysis is an excellent biomarker because it is capable of detecting genetic material lesions or malfunctioning mitotic flow caused by aneugenic mechanisms [25].

Nanotoxicity testing is critical to understanding the mechanisms and to pinpoint the potential negative effects of different types of NPs for their sustainable development in the future and for developing effective approaches to mitigate their negative effects [8].

1.3.1. Toxicity of silver nanoparticles

Exposure of the human body to silver NPs can occur through different pathways: inhalation, ingestion, injection, or physical contact through cuts or wounds. Caution is required because some *in vitro* data have shown that in some cases low concentrations of silver NPs may be toxic [4]. The cytotoxicity and genotoxicity of silver NPs are depending on concentration, dispersion, size and functionality of the surface. The reports have shown that the size of silver NPs is an important factor for cytotoxicity and genotoxicity, probably acting through mechanisms of apoptosis and necrosis [4]. For example, positive surface loading of these NPs makes them more suitable, allowing them to remain for a long time in the bloodstream compared to negatively charged NPs, which are a major vehicle for administering anti-cancer agents [26].

When investigating the biological activities of silver NPs, it is important to take into account that NPs will always interact with the protein medium, such as a cell culture medium or a bacterial culture, before their biological actions could occur [4]; the toxicity of silver NPs is closely related to their transformation into biological media, including surface oxidation, silver ion release, and interaction with biological macromolecules. Also, toxicity depends on the state of silver: metal, soluble or insoluble. It has been shown that repeated exposure to ionic silver causes anemia, cardiac enlargement, growth delay, and degenerative changes in liver in animals. Chronic exposure to humans most often leads to argyrias, an irreversible blue-gray pigmentation of the skin and / or eyes [21]. The dissolution of silver NPs may also play a key role in their toxicity.

An important mechanism of toxicity for silver NPs is the interaction of both ionic form and silver NPs with sulfur-containing macromolecules, such as proteins, due to the strong silver affinity for sulfur [27]. Silver NPs can interact with membrane proteins and activate signaling pathways, leading to inhibition of cell proliferation (Fig. 2) [27].

Silver NPs can also penetrate the cell through diffusion or endocytosis to cause mitochondrial dysfunctions, the generation of reactive oxygen species (ROS) leading to damage to proteins and nucleic acids, and ultimately to the inhibition of cell proliferation (Fig. 3) [27]. Oxidative stress occurs when ROS generation exceeds the capacity of the cellular antioxidant defense system [27]. After introducing silver NPs into cells, the first step is cell distribution, a critical event for determining toxicity. The nanoparticle absorption mechanisms include endocytosis mediated by pinocytosis and phagocytosis [26] Asharani *et al*. have shown by electronic transmission microscopy (ETM) that silver NPs can penetrate into cellular compartments such as endosomes, lysosomes and mitochondria [4] . Once silver NPs penetrate the cell, its fate is determined by many factors, including the effectiveness of antioxidant defense, the efficiency of the DNA repair system, the apoptotic trend and the cellular signaling mechanism. Oxidative damage to DNA is the ultimate cellular event of apoptosis, as evidenced by many diseases, including cancer, neurodegenerative disorders and cardiovascular disease [26].

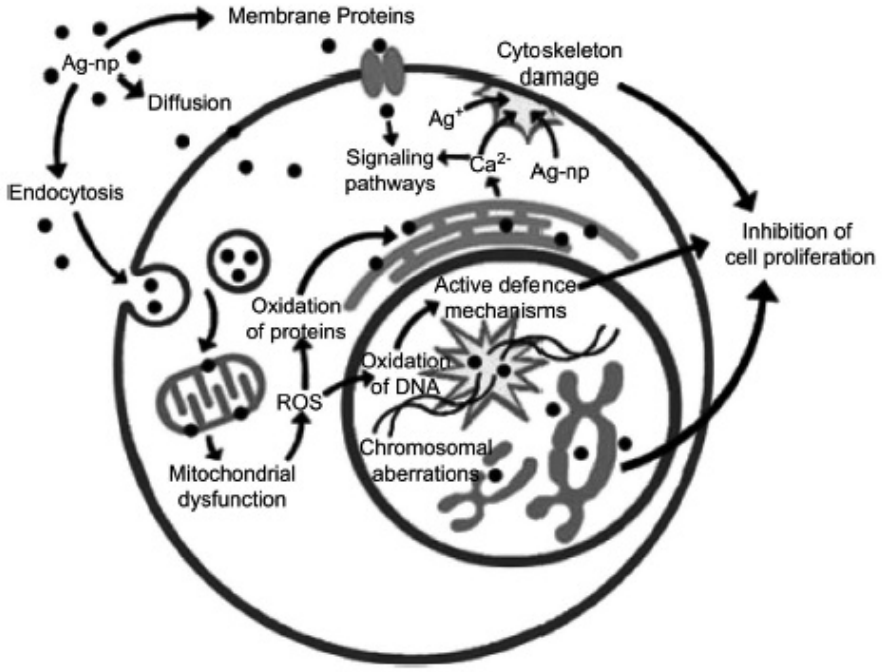

Fig. 3 - The mechanism of silver nanoparticles toxicity (McShan *et al.*, 2014)

Inhalation toxicity of silver NPs was investigated in Sprague-Dawley rats over a 28-day period. Rats were exposed to Silver NPs for 6 h a day, 5 days a week, for a total of 4 weeks. The results showed that male and female rats showed no significant changes in body weight and hematology relative to the silver NPs concentration. However, significant dose-dependent changes in alkaline phosphatase and cholesterol levels were found in male or female rats, which seems to indicate that exposure to more than 300 mg of silver NP may lead to mild liver injury. While some researchers reported that the lungs are major target tissues affected by prolonged inhalation exposure to silver NPs in another publication, Lee and colleagues reported that silver NPs exposure modulated the expression of several genes associated with motor neuron disorders, neurodegenerative disease and immune cell function, indicating potential neurotoxicity and immunotoxicity associated with silver NPs exposure. Minimal pulmonary inflammation or cytotoxicity of mice were found after 10 days of exposure to Silver NPs [28].

It has been suggested that silver NPs do not induce genetic toxicity in male and female rats bone marrow *in vivo*. There are several publications on studies on Silver NPs toxicity to the body by exposure or injection of the skin. Generally, very little work has been done on the *in vivo* toxicology of Silver NPs, and additional investigations in this area are needed to accurately assess the real impact of Silver NPs in commercial products on humans and animals. Frequent causes of Silver NPs-induced toxicity include oxidative stress, DNA damage and apoptosis [28].

A quantitative *in vivo* study was performed and low transport and Silver NPs toxicity in zebrafish were demonstrated. In this study, researchers showed that 30-72 nm diameter Silver NPs were able to diffuse into zebrafish embryos through the chorionic pores through Brownian random motion, and thus may have a stronger toxic effect. Oxidative stress and apoptosis were evaluated in zebrafish liver, and the hepatotoxic behavior of silver NPs was concluded [22].

Silver NPs induce DNA damage to different types of mammalian cells through chromosomal aberrations, DNA breaks and mutations [26].
Genotoxicity is described as lesions caused to genetic information inside a cell due to chemical agents, resulting in genetic mutation, chromosome modification, and DNA damage. Genotoxicity is a major risk factor for long-term toxic effects such as carcinogenesis [22]. The genotoxicity of silver NPs was tested on many types of cells, including red blood cells, rat hepatic BRL3A cells, PC-12 neuroendocrine cells, GSC germ line cells, MCF-7 human breast adenocarcinoma, human liver HepG2 cells, BEAS-2B bronchial epithelial cells, A4549 pulmonary alveolar epithelial cells and hMSC human mesenchymal stem cells [4]. Until now, data collected *in vitro* and *in vivo* indicate that the

production of ROS is also responsible for numerous molecular and biochemical modifications related to genotoxicity.

Epigenetics involves stable and hereditary changes in gene expression without changing the DNA sequence. Epigenetic mechanisms include mainly DNA methylation patterns, posttranslational histone tail changes, chromatin remodeling and microRNAs (miRNAs) [29]. It has been shown that silver NP may modify histone methylation, thereby causing the expression of the globin gene in red blood cells. NPs can increase the production of reactive oxygen species and oxidative damage to DNA, which can affect the ability of methyl-transferases to lead to DNA hypo-methylation and to alter the expression of methylation-regulated genes [29].

1.4.2. Toxicity of ZnO NPs

Among intentionally designed NPs, metal oxide NPs are the most used nanomaterials. It has been demonstrated that exposure to metals can often result in toxicity, modification of gene expression, epigenetic signs and metal-induced carcinogenesis [29] . Although ZnO NPs are of great commercial importance and are present in various products, there is clearly an increasing concern of the consumers to know the toxicological effects of ZnO NPs. The toxicological studies conducted on ZnO NPs over the past ten years have shown potential risks to health and to the environment. These NPs may be toxic not only to bacteria, but also to mice and human cells [7].

ZnO has been shown to be toxic *in vivo* by certain routes of exposure. For example, ZnO NPs were more toxic to the lung than the equivalent ionic strength ($ZnCl_2$) dose after intratracheal injection. It is believed that the particles are trapped in the lungs and continuously release zinc ions which cause toxicity [20].

The ZnO NPs have been shown to exhibit the ability to penetrate into the skin and reach viable cells, thus having a toxic potential. A comparative analysis of dermal penetration among different animal species was performed in several species being evaluated in the rabbit> rat> pig> monkey> human sequence. It has been observed that pig and rat skin is up to 4 and 9-11 times more permeable than human skin [7]. When using intravenous administration, absorption does not take place, so other aspects of toxicokinetics such as tissue distribution and removal can be studied more accurately. Due to the abundance of zinc in the body, ZnO radioactive NPs were used for biodistribution studies in mice [5]. Intravenous injection induced primary retention in the lung (43.6% of the injected dose per g wet weight, 1 hour after administration) and subsequent translocation to the gastrointestinal tract for fecal excretion [5].

Single intraperitoneal administration of 100 nm (2.5 g/kg body weight) ZnO NPs resulted in accumulation in the liver, spleen, lung, kidneys and heart. The zinc concentration in liver, spleen and lung was higher after nanoparticle administration than after the administration of similar amounts of Zn particles of 1 μm [5].

Oral administration of ZnO NPs at 100 nm (2.5 g/kg body weight) resulted in accumulation in the liver, spleen, lung and kidneys. Unlike intra-peritoneal administration, ZnO NPs did not accumulate in the heart. The systemic distribution of ZnO to nano- and micro-dimensions also shows after oral absorption, greater absorption and a stronger toxic effect of nanoscale particles. The liver, spleen, lung and kidneys and, in some cases, the heart are target organs [5].

Other than the inherent properties of ZnO NPs, physicochemical properties, such as surface dimensions and charges, could contribute to the toxicity of ZnO NPs. NP surface loading is also an important factor in assessing toxicity, since positively charged NPs have been shown to have higher toxicity, and differential surface load is attributed to cellular absorption and intracellular localization [19].

It has been shown that ZnO NPs toxicity could be attributed to Zn oxidation to free Zn^{2+} ions and generation of free radicals on the surface of ZnO, resulting in cellular and metabolic ionic imbalance that is associated with a defect in ionic homeostasis and inhibition of ionic transport. Toxicity studies have shown that the generation of reactive oxygen species (ROS) and the occurrence of a classical dose and time dependent ROS-induced oxidative stress response, is the mechanism leading to ZnO NPs - mediated toxicity [20]. The insufficient antioxidant protection mechanism due to the excessive production of ROS has been shown to cause cell death and genotoxicity [19].

Some toxic effects of *in vivo* exposure have been reported in vertebrates such as rodents and zebrafish, as well as in invertebrates, for example *D. melanogaster*. The studies using *D. melanogaster* did not report any toxicity observed with the ingestion of ZnO NPs. On the other hand, another prior study evaluating genotoxicity and oxidative

stress induced by ZnO NPs in *D. melanogaster* showed poor genotoxicity of ZnO NPs [19].

It was reported that sublethal concentrations of ZnO NPs can induce epigenetic changes, which can lead to reprogramming a wide spectrum of genes expression [29]. Exposure to ZnO NPs is known to primarily affect the lungs, therefore, the pulmonary fibroblast cell line (MRC5) is used as a model to determine potential modulation in DNA methylation. It has also been demonstrated that ZnO NPs exhibit a certain preferential toxicity to human myeloblastic leukemia (HL60) cancer cells compared to peripheral blood mononuclear cells. There may be several reasons for preferential cell toxicity. A potential mechanism for this behavior may involve the generation of ROS, which is generally supposed to occur after cellular absorption of zinc ions [20]. Different cells of the immune system respond differently to ZnO nanomaterials. For example, dendritic cells exhibit a dose-dependent cytotoxic response and an increase in caspase activity, enzymes involved in the death of apoptotic cells. Few studies have investigated the neurotoxicity of ZnO NPs [5]. Although ZnO NPs (60-200 nm) exhibited clastogenic activity *in vitro* in mammalian cells, there was no indication of clastogenic potential or aneugenic activity *in vivo* [5].

CONCLUSION

Among the various types of nanomaterials that have been developed with the emergence of nanotechnology, metallic and metal oxide nanoparticles are the most widely used for different types of applications in various fields of human activity. One of the main biomedical applications of silver and ZnO NPs is represented by the development of novel antimicrobial strategies. However, their higher reactivity at atomic scale size could also increase the risk of toxicity for live organisms which are exposed directly or indirectly to them.

Therefore, further studies are required in order to elucidate the intimate mechanisms of their toxicity on standardized *in vitro* and *in vivo* models, the factors influencing their cytotoxicity degree (size, form, chemical composition, aggregation, solubility, route of administration, dosage, exposure duration, bioaccumulation, metabolism etc.). These nanotoxicity assays are critical for both achieving a sustainable development in the future and for developing effective approaches to mitigate their negative effects.

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FISH TANK GRANULOMA – A CHALLENGING DIAGNOSIS

Mădălina Mitran[1,2], Cristina Mitran[1,2], Mircea Tampa[*1,2], Ana Maria Pîrvan[2], Isabela Sârbu[1], Clara Matei[1], Rodica Trifu[2], Mircea Ioan Popa[1,3], Simona Roxana Georgescu[1,2]

[1]*Carol Davila University of Medicine and Pharmacy, Bucharest, Romania*
[2]*Victor Babes Clinical Hospital for Infectious Diseases, Bucharest, Romania*
[3]*Cantacuzino National Research Institute, Bucharest, Romania*

ABSTRACT

Non-tuberculous mycobacteria (NTM) are widespread in the environment and most of them are non-pathogenic for humans, but some species may be pathogenic and sometimes cause severe conditions. The pathology caused by NTM primarily affects the respiratory system and skin.

Patients susceptible to the disease are those with a deficient immune status and rarely those immunocompetent. *Mycobacterium marinum* is a non-tuberculous mycobacterium, with a slow multiplication rate, which produces especially cutaneous infections and its main habitat is the aquatic environment. *M. marinum* requires special cultivation conditions, making its isolation difficult.

We present the case of a 47-year-old patient who addressed our clinic for violaceous nodules located on his right hand after the cleaning of a fish tank.

REZUMAT

Mycobacteriile netuberculoase (NTM) sunt larg răspândite în mediu, dar majoritatea sunt nepatogene pentru om, totuşi unele specii pot fi patogene şi pot cauza uneori afecţiuni severe. Patologia cauzată de NTM afectează în principal sistemul respirator şi pielea.

Pacienţii susceptibili la boală sunt cei cu un status imun deficitar şi mai rar cei imunocompetenţi. *Mycobacterium marinum* este o NTM, cu ritm lent de multiplicare, care produce în special infecţii cutanate şi habitatul său principal este reprezentat de mediul acvatic. Necesită condiţii speciale de cultivare, ceea ce face dificilă izolarea sa.

Prezentăm cazul unui pacient în vârstă de 47 ani, care s-a prezentat în clinica noastră pentru noduli violacei localizaţi la nivelul mâinii drepte, apăruţi după curăţarea unui acvariu.

Keywords: *Mycobacterium marinum,* skin infection, fish tank granuloma, diagnosis

INTRODUCTION

Non-tuberculous mycobacteria (NTM) are ubiquitous microorganisms, being encountered in water, soil, plants, and animals, and proved to be opportunistic pathogens for humans. Under certain conditions, these microorganisms can cause different infections in humans, including pulmonary, osteoarticular and cutaneous infections [1]. Most commonly, the cutaneous infections are caused by *M. marinum* and *M. ulcerans*, the etiologic agents of fish tank granuloma and, respectively Buruli ulcer [2]. In Europe, *M. marinum* is the most common mycobacterium involved in cutaneous infections, whereas in tropical areas, *M. ulcerans* is the most frequently isolated [3].

M. marinum was isolated for the first time by Aronson in 1926 from a fish [4], and in 1954 Linnel and Norden described the swimming granuloma, they establishing the link between *M. marinum*, formerly called *M. balnei* and that cutaneous manifestation [5]. *M. marinum* is a slow-growing NTM that forms yellowish-beige colonies on Lowenstein Jensen medium at 25-35º Celsius. Infection occurs through contact with infected water from lakes, rivers, swimming pools or aquariums.

In most cases, the infection has been identified in 38-45 years old adults who keep fish tanks or have fish-related occupations [6]. The main vectors are freshwater fish, dolphins, shrimps, oysters and snails. Most commonly there is a skin injury that represents the site of inoculation [7].

Clinically, the infection manifests as a solitary erythematous and exudative nodule, but can also appear as papules or ulcerated plaques, with an incubation period of about 3 weeks [8]. It was reported that the infection can disseminate predominantly in immunocompromised hosts and lead to arthritis, synovitis, osteitis or pulmonary disease [9]. The gold standard for diagnosis is the isolation of the microorganism, but this is often difficult, mycobacteria requiring special conditions of cultivation. Thus, anamnestic, clinical and

**Corresponding author:* Mircea Tampa, tampa_mircea@yahoo.com

histopathological data play a pivotal role in establishing the diagnosis [10].

CASE PRESENTATION

A 47-year-old male patient, living in the urban area, with no significant medical history presented in our clinic for violaceous nodules on the dorsal face of his right-hand. The nodules were 3 months old and their evolution was expanding. Suspecting a bacterial infection his medical physician recommended him oxacillin, but without any improvement.

From the patient's history we noted that he owned a fish tank that he regularly cleaned without using gloves. The physical examination revealed violaceus nodules of various sizes with fluctuating consistency and centripetal distribution, located on the dorsal part of the hand and on the posterior forearm (Fig. 1). The nodules were slightly painful upon palpation and the patient reported having pain before the appearance of any visible lesion. He also stated that within a few months, prior to the occurrence of the nodules, the distal phalanx of the 4th finger developed a small ulceration area. Following the anamnesis and clinical examination, a suspicion of fish tank granuloma was raised.

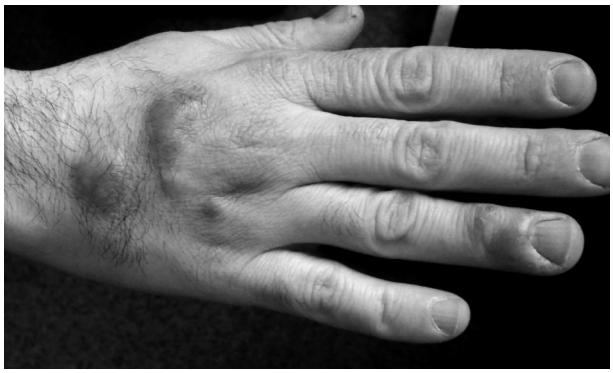

Fig. 1 – Violaceus nodules of various sizes with fluctuating consistency and centripetal distribution, located on the dorsal part of the hand

The usual blood tests were in normal parameters. A biopsy of one of the nodules was performed which revealed the presence of a non-specific granulomatous process.

In the dermis was observed a significant inflammatory process, consisting of lymphocytes, histiocytes, granulocytes, eosinophils, nuclear fragments and necrosis. Microorganisms were not detected. Samples for bacterial and fungal cultures were also taken (Fig. 2). The Ziehl Neelsen stain was negative. Cultures were carried out in both usual

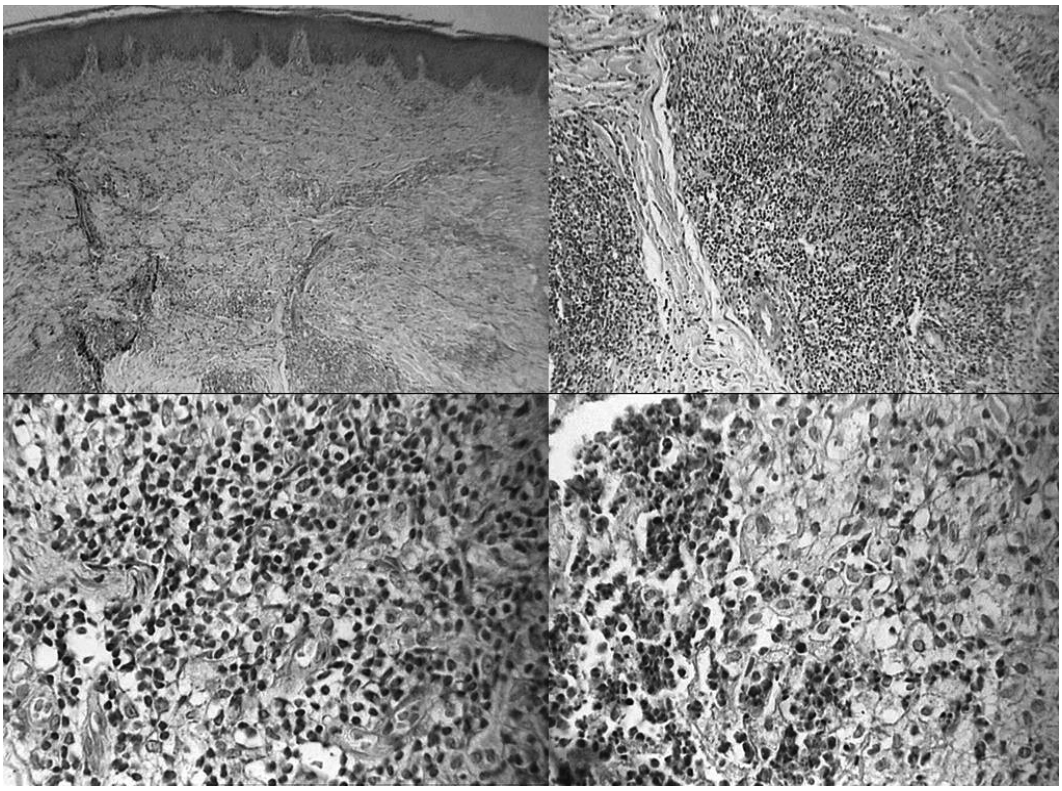

Fig. 2 - Histopathological aspect, hematoxylin and eosin stain - inflammatory infiltrate consisting of lymphocytes, histiocytes, granulocytes, eosinophils, nuclear fragments and necrosis

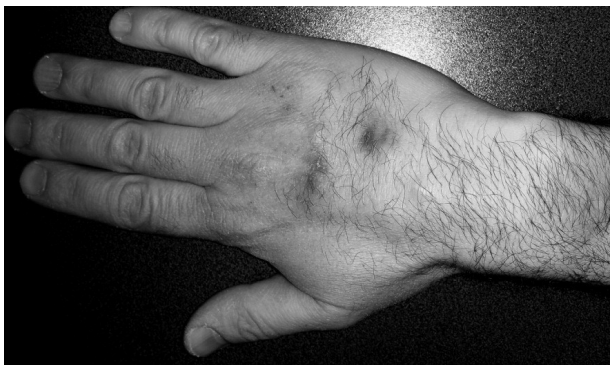

Fig. 3 - Clinical aspect after 3 months of treatment – active lesions still persist

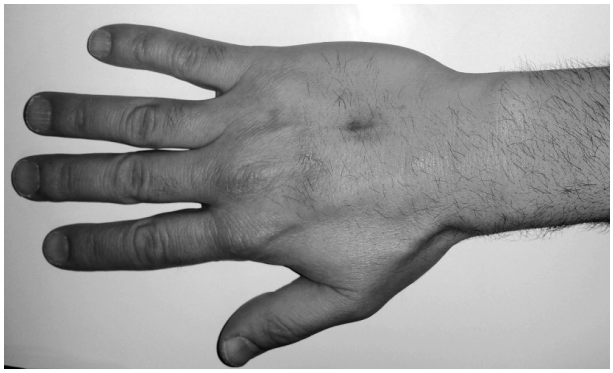

Fig. 4 - Clinical aspect at the end of the treatment – residual hyperpigmented patches

medium and also in special Mycobacteria medium, but they were found to be negative.

Based on the anamnestic, clinical and histopathological aspects we established the diagnosis of fish tank granuloma. The patient was treated with clarithromycin 1g per day.

Evolution was favorable with the involution of the nodules, but with a persistence of small nodules on the lymphatic tract, possibly due to greater difficulty of the antibiotic to penetrate at this level (Fig. 3). After 3 months of treatment, we decided the continuation of the antibiotic therapy for another 3 months. At the end of the 6 months of treatment, we could observe the involution of all the nodules (Fig. 4).

DISCUSSION

Mycobacterium marinum is a non-tuberculous photocromogenic mycobacterium, which pertains to group 1 according to Runyon's classification [11] and its natural habitat are both the fresh and salt water [12]. The skin infection caused by *M. marinum* is called fish tank granuloma or swimming pool granuloma, depending on the source of the infection [13]. The incidence of fish tank granuloma varies between 0.04 and 0.27 per 100,000 inhabitants, but some cases remain unreported [14]. Usually the incubation period is about 2-4 weeks and ranges between 2 and 9 months as indicated in medical literature [15, 16].

The main way of transmission is the contact with infected water [17], in the context of a previous skin trauma [18]. It has been noticed that before 1960 a higher number of cases of infection were reported compared to the subsequent period. This may be due to the improvement of water chlorination in swimming pools [19]. Cases of infection with *M. marinum* after contact with coal mine water have also been described [20]. A study that analyzed the reported cases in medical literature during 1962-1996 found that almost half of them (49.2%) were linked to an aquarium and nearly a third were related to injuries caused by fish or shellfish (27.4%) [19]. In our case, most probably the infection source was the fish tank, the patient reporting that he regularly cleaned it without gloves and the site of inoculation was the small ulceration on his 4th finger.

In most cases the lesions are located on the upper limbs [21]. The lesions are located on the lower limbs, especially when the source of infection is a swimming pool [22].

The presence of lesions predominantly on the extremities can be explained by the fact that mycobacteria prefer lower temperatures and therefore disseminated infections rarely occur [23, 24]. However, there are some particular strains of *M. marinum* that grow at 37º Celsius [17].

Sporotrichosis is an important differential diagnosis considering the fact that the lesions may have a sporotrichoid aspect, appearing along the lymphatics in 20 to 40% of cases. Adenopathies are not commonly observed [23, 25, 26]. A study that investigated 17 cases of skin infections caused by NTM, identifying *M. marinum* as causative agent in 14 of them, highlighted that many patients had atypical clinical presentations such as herpetiform, ulcerative or bruise-like lesions [1].

Deep tissue penetration may occur, being reported cases of tenosynovitis, osteomyelitis, or soft tissue necrosis, which can result in the amputation of the affected segment; such cases were more frequently diagnosed in immunosuppressed patients [21, 27, 28]. An analysis of the cases of disseminated infection reported in medical literature has revealed tenosynovitis as the most common complication [28].

The rarity of the infection in current medical practice and the slow growth rate of *M. marinum*, that implies apparently negative cultures during the usual examination period, are factors that often contribute to a delayed diagnosis [29]. In many cases, the disease is not recognized and is mistaken for conditions such as fungal infections, cellulitis, rheumatoid arthritis or cutaneous tumors. Unfortunately, a delayed diagnosis is associated with a higher risk of complications [30]. Thus a detailed anamnesis plays a very important role and must include even the hobbies of patients, in our case of interest being the possession of a fish tank. The clues that may raise the suspicion of an infection with *M. marinum* are socio-occupational factors such as fish-related activities and the presence of a previous cutaneous injury [10, 31].

Ang and colleagues examined 38 cases of *M. marinum* infection, diagnosed on anamnestic, clinical and histological criteria. They found a positive smear for acid fast bacilli (AFB) in the case of only 13.2% [5] of patients and the isolation of the microorganism in cultures was obtained only in one case of the 35 analysed (2.9%) [10]. The identification of *M. marinum* in cultures varies between 2.9 and 96.5%, the average time for positive cultures being 24 days [6]. In the study by Ho *et al.*, 76.5% of patients had positive cultures, but AFB were identified in a low percentage. It seems that a higher number of bacteria are in the exudative lesions, compared to the granulomatous lesions, in which the number is reduced, and in that study all the lesions were granulomatous [32]. In our case the Ziehl Neelsen stain was negative and we could not isolate the bacteria. However, we should take into consideration that in general the samples are paucibacillary and the microorganism is difficult to cultivate and in this case a negative culture does not rule out the diagnosis of NTM infection.

Negative cultures using common cultivation techniques and lack of response to classic antibiotic treatment are important diagnostic indices [33]. *M. marinum* requires special cultivaton conditions.

The optimal cultivation temperature is 30-33º Celsius and a positive culture is obtained in 7-10 days. A faster diagnosis is achieved using the PCR technique [23].

The histopathological examination plays an important role in differential diagnosis. In the early stages of infection, signs of nonspecific inflammation, including primarily an infiltrate consisting of lymphocytes, neutrophils and histiocytes, associated with hyperkeratosis and acanthosis, are observed. In advanced stages tuberculoid granulomas, usually without caseification are noticed [26]. The presence of granulomas is a strong argument in favour of a mycobacterial infection. Microorganisms are rarely seen [30]. In most cases, the infection does not invade the dermis, the involvement of subcutaneous tissue being reported especially in sporotrichosis like lesions [23]. In our case, the histopathological examination was an important argument in favour of a granulomatous infection, but microorganisms could not be revealed.

In immunocompetent patients, the infection is self-limiting and the lesions may heal after several months or years, but often the resolution is accompanied by scar formation [24, 25]. Given the small number of cases and lack of clinical trials, there is no standardized treatment [26]. The therapy is based primarily on antibiotic treatment. Among the antibiotics that may be used are ethambutol, rifampicin, clarithromycin or doxycycline. Other therapeutic options reported as effective are surgery, cryotherapy, electrocautery, radiotherapy or photodynamic therapy [22, 33].

Classical antibiotics such as isoniazid or pyrazinamide, which are the basis for TB treatment, should be avoided, *M. marinum* being resistant to them [33]. A national survey in France that included 63 cases of *M. marinum* infection confirmed by positive cultures revealed a mean duration of treatment of 3 and a half months and the most commonly prescribed antibiotics were clarithromycin, cyclins and ethambutol [12].

In cases of superficial infection, monotherapy with clarithromycin appears to be effective and in cases of deep infection it is recommended to associate ethambutol and rifampicin in combination with surgery. In fact, there is no clear evidence of the role of surgery, this method being used mainly in deep infections [18, 21, 33]. Some researchers recommend continuing treatment for 1-2 months after the resolution of the lesions [21], while in cases of deep infection, treatment should be given for at least one year [34]. Topical corticosteroids should be avoided, as there were cases when the worsening of the disease has been reported after their use [25]. Sette in his case of a long standing infection with *M. marinum* in an immunocompetent patient has achieved remission of lesions after 3 months of clarithromycin treatment [14]. We decided a prolonged treatment with clarithromycin, because after 3 months of therapy small active lesions still persisted.

CONCLUSIONS

In the current medical practice, *M. marinum* skin infections are rare, therefore a high level of suspicion is needed; awareness of the main risk factors and clinical aspects are very important. In addition, the diagnosis is difficult, special conditions for isolation being required. It should be taken into consideration that in a significant percentage the cultures are negative and evocative anamnestic, clinical and histopathological aspects can establish the diagnosis.

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OVERVIEW ON TOXOCARIASIS: CLINICAL AND LABORATORY DIAGNOSIS IN PRESUMED CASES WITH NEGATIVE ELISA

Carmen Michaela Crețu[1,2,3], Mădălina Preda[1], Patricia Mihăilescu[3], Florin Popescu[1,4], Camelia Elena Berghea[1,5], Tania Cristodulo[3], Gabriela Loredana Popa[1,2]

[1]Carol Davila University of Medicine and Pharmacy, Bucharest, Romania
[2]Colentina Clinical Hospital, Parasitology Clinic (CDPC), Bucharest, Romania
[3]Eco-Para-Diagnostic SRL Bucharest, Romania
[4]N Malaxa Clinical Hospital, Allergology Clinic, Bucharest, Romania
[5]Marie Curie Hospital, Allergology Departament, Bucharest, Romania

ABSTRACT

Toxocariasis is a parasitic zoonosis caused by the genus *Toxocara,* for which sometimes the diagnosis can be difficult. We retrospectively studied 91 consecutive cases of toxocariasis, during 2015-2017. The majority of the patients included in our study belonged to the age group of 31-40 years, and women were the most affected. Regarding their symptoms, the most frequent one was pruritus, followed by asthenia, myalgia, lymphadenopathy, fever, wheezing and others.

In the present study, for the diagnosis of ocular or visceral toxocariasis we used ELISA assay employing TES (*T. canis* excretory – secretory antigens) harvested from *T. canis* larvae and confirmation with WB commercial kits. We used indirect ELISA to determine the prevalence of total IgG antibodies against TES. In characteristic symptomatic patients, with suspicion of toxocariasis, ELISA tests were negative for 73 (80%) patients out of 91. We also performed Western Blot, which was positive for all the patients. For IgG avidity test performed for a part of the patients, the results indicated that 83% of them had an old infection.

Albendazole was the only molecule used as anti parasitic agent for the study group. Antiallergic medication, non-steroids anti-inflammatory or corticosteroids were added to diminish the clinical signs or to prevent the possible side effects of the treatment.

The most important conclusion of our study is the impact of Western Blot in the diagnosis of toxocariasis, in the context of characteristic symptoms and negative ELISA.

Keywords: toxocariasis, Western Blot

REZUMAT

Toxocaroza este o zoonoză parazitară cauzată de genul *Toxocara*, în care uneori diagnosticul poate fi dificil. Am studiat retrospectiv 91 de cazuri consecutive de toxocaroză, în perioada 2015-2017. Dintre pacienţii incluşi în studiul nostru, majoritatea au fost în grupa de vârstă de 31-40 de ani, iar femeile au fost mai frecvent afectate. În ceea ce priveşte simptomele, cel mai frecvent a fost pruritul, urmat de astenie, mialgie, adenopatie, febră, respiraţie şuierătoare şi altele.

În studiul prezent, pentru diagnosticul de toxocaroză oculară sau viscerală, am utilizat testul ELISA utilizând TES (antigene excretoare-secretoare *T. canis*) procesate din larve de *T. canis*. Am folosit metoda ELISA indirectă pentru a determina prevalenţa anticorpilor IgG total, împotriva TES. Rezultatele testului ELISA au fost negative pentru 73 (80%) din 91 de pacienţi. În contextul simptomatologiei specifice, am efectuat, de asemenea, Western Blot, care a fost pozitiv pentru toţi pacienţii. Pentru testul de aviditate IgG, efectuat pentru o parte din pacienţi, rezultatele au arătat că 83% dintre aceştia au avut o infecţie veche.

Albendazolul a fost singura moleculă pe care am folosit-o ca agent antiparazitar pentru grupul de studiu. Au fost adăugate medicamente antialergice, antiinflamatoare nesteroidiene sau corticosteroizi pentru diminuarea semnelor clinice sau pentru prevenirea posibilelor efecte secundare ale tratamentului.

Cea mai importantă concluzie a studiului nostru este impactul pe care Western Blot îl are în diagnosticul de toxocaroză, în contextul simptomelor caracteristice şi test ELISA negativ.

INTRODUCTION

Toxocariasis is a parasitic zoonosis produced by genus *Toxocara* (more than 30 species), with worldwide morbidity. Most common and important for humans are *Toxocara canis* and *Toxocara cati* [1, 2].

A child could be accidentally infected by ingestion of eggs from *T. canis* (hands or hair of the dog contaminated through puppies contact) [2-4], or by ingestion of vegetables or soil contaminated with *Toxocara* eggs [2, 4-6]. Consumption of raw liver from *Toxocara*-infected paratenic hosts is a less frequent source of contamination [2, 4, 6-9].

Toxocariasis is an important public health problem; seroprevalence studies have shown

*Corresponding author: Carmen Michaela Crețu; Email: michaelacarmen.cretu@gmail.com

that this disease is more frequent among children from socio-economically disadvantaged populations [2, 6, 10].

MATERIALS AND METHODS

We present a retrospective study, with the analyses and interpretation made for the data collected from 91 consecutive infected patients, confirmed by Western Blot (WB), for the diagnosis purposes of ocular toxocariasis (OLM) and visceral toxocariasis (VLM). The study period was between September 2015 and April 2017. The information was gathered from 91 infected patients with either ocular or visceral toxocariasis admitted to the Parasitology Department in Colentina Hospital, or in Eco-Para-Diagnostic Medical Centre. The study will discuss the clinical relevance, the metodology, the optimal therapy in each case as well as the evolution and the prognosis. Taking into account that most of the patients were referred to parasitology from allergology, we focused on the peculiarities of those patients. It was a strong cooperation with the Ophthalmology Department in the University Emergency Hospital, Bucharest, Allergology Department in Elias Hospital, Budimex Pediatric Hospital, Malaxa Hospital and the Dermatology Department in Colentina Clinical Hospital.

The data obtained from the files of the patients were analyzed from different perspectives: epidemiology, clinical forms/syndromes, diagnosis, treatment, evolution and prognosis.

We organized the information gathered from patients in the following categories:

- information about the patient: gender, age, location (rural/urban), house pet or veterinary, hygiene, meals;
- clinical importance: pruritus, allergies and angioedema, dry cough, wheezing, fever, asthenia, myalgia, adenopathy, fever, ocular symptoms, abdominal complaints, etc;
- blood work: leukocytes, immunoglobulins, LDH, CK, NL and eosinophils, total IgE;
- serology: ELISA, WESTERN- BLOT, Avidity index for *Toxocara* (AI);
- imaging: ultrasound, X ray, CT, IRM.

RESULTS AND DISCUSSION

Age: The collected data in our study showed the age distribution among 91 patients. The largest age group was between 31-40 years of age with maximum number of cases 23 (25%). Gender distribution shows more female patients infected with *Toxocara* spp, namely 52 (57%) as compared to the male patients - 39 (43%). The higher number of cases in adult population, unusual if compared with the literature data, can be explained by the fact that most of the patients referred in Colentina Hospital are adults. There is not a pediatric department in the hospital.

Area of distribution observed and analyzed. A higher number of patients came from urban areas, 70 patients (77%), as compared to the rural patients, 21 (23%). Interestingly, more patients infected with *Toxocara* spp belong to the urban life. Many of them do not recognize the contact with dogs or cats. The explanations we can draw are related to the fact that many of them are travelling to the countryside during the weekend and the presence of the stray dogs in urban areas. Clearly, the socio-economic aspects play a great role here. On the other hand, the urban population has a higher access to medical facilities.

Risk factors and symptoms

The results in this study show that the majority of the patients, 58 out of 91, have involved contact with dogs, (64%). The next major group were patients that did not know the reason why they got affected, 27 (30%). A small group of patients claimed they had a contact with a cat, 3 (3%). The rest of the results show for hygiene, veterinary, agriculture (1% each) (Fig. 1).

Our patients were complaining and seeking treatment for their initial symptoms. Out of 91 patients, 72 (77%) complained about pruritus

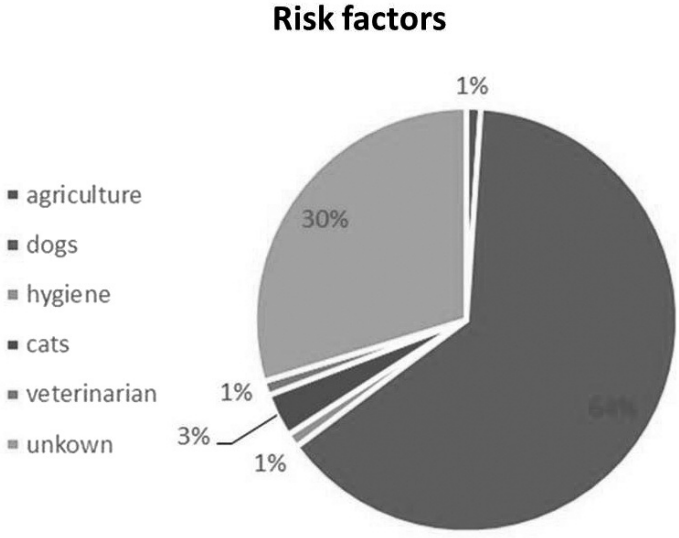

Fig. 1 - Distribution of patients according to the risk factors

(intense itching). Sixty three patients presented asthenia (68%). Other signs and symptoms were: myalgia – 56 (60%), lymph node enlargement – 20 (22%), fever – 19 (20%), wheezing – 18 (19%), cough – 11 (12%), decreased visual acuity, or blurred vision – 17 (18%), irritability – 8 (9%), somnolence – 5 (5%), tremor – 2 (2%), paresthesia – 4 (4%), headache – 5 (5%), dementia – 1 (1%) (Fig. 2).

It is easy to notice that most of the patients complained about persistent pruritus and allergies, asthenia and myalgia. But, also, the most important clinical signs and symptoms were mentioned by the patients, including ocular and neurological problems, rare locations of the parasite.

Since for many patients different types of allergic reactions were noticed, a careful systematic screening for *Helicobacter pylori*, thyroid function, food and respiratory allergies tests, autoimmune disorders, level of diaminoxidase (DAO), other possible parasitic diseases was done, in collaboration with allergologists.

Severity of the cases and associated infections:

From all 91 patients in our study, we observed 3 severe cases which divided in *visceral larva migrans* (VLM) 2 (2%) cases and *ocular larva migrans* (OLM) 1(1%). All the other 88 (96.7%) cases were considered as mild. Based on Avidity index, only VLM seemed to be re-infection of the patients with a total number of 6 (6%) out of 91. The final diagnostic results showed 7 (7.7%) patients that presented *covert toxocariasis* (CT), 67 (73.62%) patients presented typical clinical VLM and 16 (17.58%) with OLM. There was 1 (1%) patient with both ocular toxocariasis and visceral toxocarisis, a less common situation. The most outline associated diseases in this study (Fig. 3) were: arterial hypertension (AHT) 6, chronic asthma/bronchitis 4, Trichinella 4, Lyme disease (including *Borrelia garinii*) 6, chronic hepatitis 7, retinal granuloma 3 (other than toxocariasis), increased liver enzymes 5, toxoplasma 3, ascaris 5, dilated cardiomyopathy 1, autoimmune thyroiditis 2.

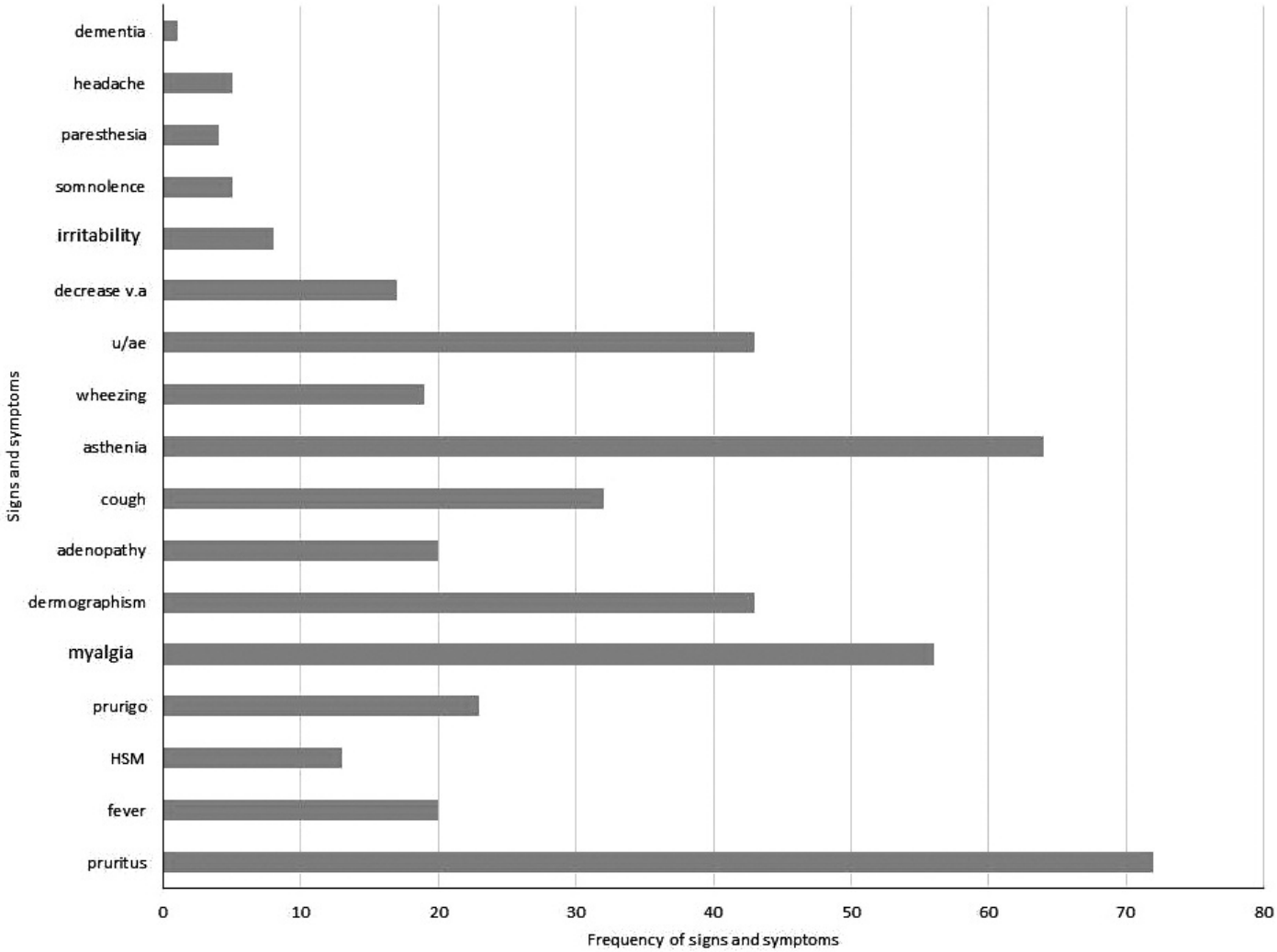

Fig. 2 - Distribution of main clinical signs and symptom

Other relevant parameters are liver and muscle enzymes (mostly creatin kinases, CK), and total IgE. Moreover, imaging techniques can complete the diagnosis.

Diagnostic approach: for the diagnosis purposes and ensuring the diagnosis in our study, we performed several tests including complete blood count, imaging and serological assays. For the hemogram, we draw attention to the full WBC count and the eosinophils level, total immunoglobulin E, muscle enzymes CK and LDH.

The eosinophils percentage was categorized for our convenience in three groups: (i) under normal limit <5%; (ii) moderate increase 5-10%; (iii) severe increase >10% (Fig. 4).

We can assume that many cases had a delayed diagnosis, showing old infections, because of the normal values of eosinophils. On the other hand, if we consider that many patients came from allergology and received antiallergic treatment or corticosteroids, which were imposed by the clinical course of the disease, we can conclude that in some cases, the eosinophil level was hidden by the symptomatic treatment

Total IgE can be increased by the parasitic trigger. In most of normal patients, non-atopic, the reverse to normal level of this parameter was noticed. For the muscle enzymes, checked in 30 patients, it seemed that CK levels were more often modified than LDH: 20 of them had normal values and 10 had an increased value for CK, while only 9 showed increased values for LDH.

Diagnostic immunology

ELISA test is a serological assay that can be used for the diagnosis of either ocular or visceral toxocariasis. The standard serological test for confirming toxocariasis is an ELISA assay employing TES (*T. canis* excretory-secretory antigens) harvested from *T. canis* larvae. The use of TES antigens from larvae increased the sensitivity and

Associated diseases	Number of patients	Percentage (%)
AHT	6	13%
Chronic asthma/bronchitis	4	8%
Lyme disease (including *B. garinii*)	6	13%
Trichinellosis	4	8%
Chronic hepatitis	7	15%
Retinal granuloma	3	6%
Increased liver enzymes	5	10%
Toxoplasmosis	3	6%
Ascariasis	5	10%
Dilatative cardiomyopathy	2	4%
Autoimmune thyroiditis	1	2%

Fig. 3 - Comorbidities associated with toxocariasis

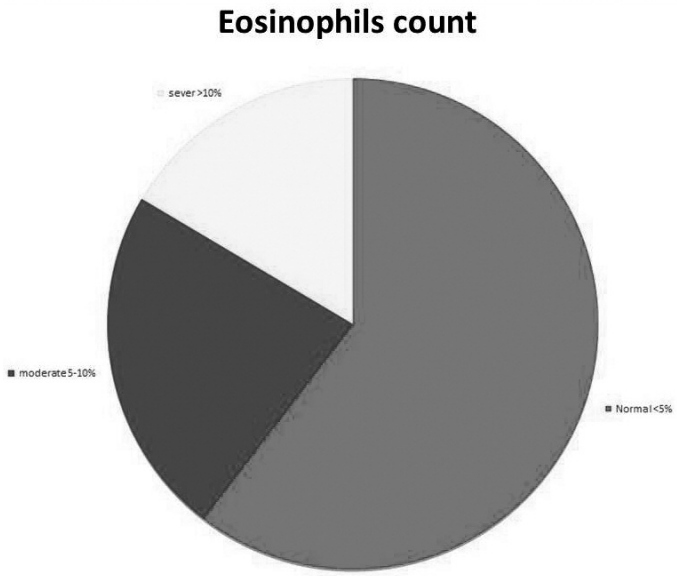

Fig. 4 - Eosinophils count on 91 patients with toxocariasis

specificity of the ELISA test compared to antigen mixtures from adult worms, although some cross-reactions with other nematodes can occur. Indirect ELISA was used to determine the prevalence of total IgG, antibodies against TES. In our study, the results of the ELISA test showed that a large group of patients had negative results: 73 out of the 91 (80%) patients with a negative ELISA test, 13 (14%) positive results and 5 (5%) tests were inconclusive.

In most of the cases with rare locations (eye, central nervous system), serology by ELISA remained negative, in spite of the very suggestive clinical appearance.

ELISA tested negative for the majority of our patients, but considering the right symptoms, relevant for toxocariasis suspicion, we performed for the same sera, using commercial **WB test,** which is more sensitive and specific than ELISA. WB showed four prominent bands representing immune-reaction of *T. canis* adults. In many studies, a 55-66 kDa antigen complex has been shown to be responsible for the cross reactivity between *T. canis* and *A. sum* [11]. The band of 81 kDa antigen was responsible for the strong cross-reactivity between *T. canis* and *Ascaris* extracts. TES antigens for diagnosing VLM on WB showed protein bands at 24, 28, 30, 35, 132, 147 and 200 kDa. In our study, Western blot showed 100% positive results (91 out of 91 patients). The results showed the specific bands of 120, 32, 26, and 35 and 24 kDa. Many patients received corticosteroids or anti allergic medication. These could explain, in part, the false negativity of ELISA in such categories of patients. Consequently, we can conclude that ELISA test has a limited value in this category of patients. In case of relevant clinical signs and symptoms for toxocariasis and negative ELISA, if all the other differentials were excluded, using of WB can be recommended. We underline that the observation that many ELISA negative patients were confirmed by WB, was the starting point of our retrospective study. We checked and analyzed all the consecutive cases with positive WB. In different studies, the results of ELISA were positive in all the cases [12], or were positive in 68% of the cases [13]. Magnaval (1989) evaluated serology by ELISA in CT patients and was positive in all the cases.

IgG avidity test

In general, it is considered that blood eosinophilia combined with positive serological tests indicate active toxocariasis requiring treatment. However, antibody levels cannot be used to discriminate between recent and chronic infections. Using anti *Toxocara* IgM, was, in our experience, associated with cross reactivity, false positivity and we did not base our diagnosis on this test. More than that, specific IgM antibodies can last up to one year, or even longer. The exact determination of the time of infection is not possible in toxocariasis because the incubation period of toxocariasis may range from weeks to months, depending on infection intensity, reinfection and patient's sensitivity. Therefore, detection of anti-TES IgG avidity in human toxocariasis is useful for determining recent or old infection, active or not active infection.

We performed IgG avidity test on 23 of our patients confirmed by serology, but with normal eosinophil level. Out of these, 19 (83%) found to show an old infection and 4 (17%) a recent infection. We analyzed the results and the severity of the infection, according to the clinical evolution: mild/severe VLM, OLM, CT. Therefore, we stratified the cases in old and mild VLM 11 (48%) patients, recent and mild infection VLM 4 (17%) patients, old and severe VLM 2 (9%) patients, old and mild OLM 2 (9%) patients, old and severe OLM 1 (4%) patient, recent and mild infection OLM 1 (4%) patient, old and mild CT 2 (9%) patients. By means of avidity tests, we established that 5 patients, having a history of toxocariasis, were re-infected (low avidity test and significant clinical signs, history of VLM). The estimation of past or recent infection in toxocariasis patients can facilitate the decision regarding the usefulness of a new therapeutic course. Of course, the number of our

series of cases is very limited, further studies being necessary in the future. A special attention should be paid to allergic patients, when screening, serological tests can have a limited value. Another way of discovering, or completing the diagnosis of VLM, CT, or OLM is by imagistic evaluation. Abdominal or ocular ultrasonography (US), abdominal, thoracic or brain computer tomography and/or magnetic resonance imagistic and X-ray are the most used techniques, showing the presence of *Toxocara* larvae in different parts of the body. In our study, most of the patients went through a US (51%) and an X-ray (38%). We noted each infection with its appearance in the image.

We categorized as follows VLM - normal appearance - 24 patients and modified in 22 cases (nonhomogeneous liver, hepatomegaly). OLM was associated with hepatomegaly in 2 patients and was normal in other 2 patients. The same description was done with the diagnostic results of chest X-ray imaging. As mentioned above, we examined 35 patients' X-ray and noticed: VLM with normal results (20); VLM with pleurisy (2); VLM with interstitial pneumonia (7); OLM with normal results in 3 cases, while the others had no characteristic changes.

Therapeutic approach

The etiologic treatment for toxocariasis infection includes different molecules: benzimidazole derivates (albendazole, mebendazole, thiabendazole), diethylcarbamazine, or ivermectin. In our study, all the patients received albendazole (ABZ) treatment, in the dosage of 10-15 mg/kylo/day, divided into two dosage/day, with silymarin and fatty meals (to increase its absorbtion), for a duration between 14 and 21 days. Patients also received anti-allergic or corticosteroids in combination with ABZ, to avoid the possible side effects and complications. As for the associated diseases, the treatment was continued.

If ocular or central nervous system location of *Toxocara* larvae was suspected, corticosteroids were initiated few days before ABZ and ABZ was started with small dosage, increased progressively. Surgery remained for complicated cases of OLM.

Prognosis

The prognosis of the cases was, generally speaking, good, mostly in VLM. The stationary evolution of the brain or ocular lesions was noticed. Irreversible complications with loss of vision were also remarked. The evolution and prognosis regarding the patients in this study seem promising with the given treatment. The overall patients with visceral, ocular or covert toxocariasis are getting improved; in other words, 55 (60%) patients out of 91 had an improvement, 29% showed neither improvement nor regression of the lesion and 10% of the patients had no compliance. The study was carried out following the clinical observation and case confirmation. The limit of the study is linked to the fact that a small number of cases were evaluated. Further prospective studies are necessary to complete the data and clarify the suspicions and observations aroused.

CONCLUSIONS

Most of toxocariasis infected patients came from the urban life (77 %) and the risk factors, the way humans become infected with *Toxocara spp,* are high when dogs, especially puppies, are involved in humans' life. The presence of the stray dogs in urban areas is not at all a neglected risk factor.

With respect to the clinical appearance, in our study, VLM syndrome was the most common (74%), most notable and was mild in its appearance. However, all the clinical forms were noticed, including OLM, CT, or cerebral lesions. Skin disorders (79%) related to toxocariasis seem to be very common and present in adult population. Special attention should be given to this category of patients. OLM is usually an independent disease, with a very high risk of sequels, even if the treatment is rapidly and correctly initiated.

Diagnosis of toxocariasis is based on clinical signs, and confirmed by laboratory tests. Positive serology either by ELISA or WB is always positive. Our diagnosis was based on the serological ELISA test using total IgE and specific IgG TES antigen. Unfortunately, ELISA results were negative in most patients, thus, using then WB testing enabled us to ensure the diagnosis of toxocariasis. This is one of the clues of our study: in symptomatic patients, negative ELISA should be completed with WB performed on sera, ocular fluid, or spinal fluid, for confirmation.

Avidity tests remain very important to confirm re-infection (in patients with history of toxocariasis) and to make the difference between old and recent, still active infections and consequently the opportunity of a new therapeutic course. Other parameters, like hyper eosinophilia, as diagnosis marker for toxocariasis, in our study, showed little relevance on our diagnosed patients, 60% having

normal values. But it should be taken into consideration that many patients receive corticosteroids for allergies, ocular or central nervous system damage and this medication can hide the eosinophil level.

ABZ was the treatment given to our patients and showed satisfying results. We chose to give ABZ 200 or 400mg/tablet, in a dosage of 10-15mg/kylo/day, for a duration of 14-21 days for most of our patients, visceral, ocular or covert toxocariasis. The patient's health was improved by the end of the therapeutic period (60%).

The evolution of most of the cases is good, or stationary, but sequels can remain for the whole life of the patient, mostly in ocular and cerebral locations. The prognosis of the cases is related to the location of *Toxocara* larvae, being very good in VLM and reserved in OLM.

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THE PREVALENCE OF DIABETES AND OBESITY IN PATIENTS WITH PERIPHERAL TROPHIC DISORDERS DETERMINED BY VEIN THROMBOSIS OF THE LOWER LIMBS

G. Trucă*[1,2], F. Popa[2,4], R.A. Macovei[2,3], G. Paunică-Panea[1,4], M.L. Fulga[1], G. Grădişteanu Pîrcălăbioru[5], C. Marcean[1,2]

[1]*Fundeni Sanitary Post High School -Bucharest, Romani;*
[2]*Carol Davila University of Medicine and Pharmacy, Bucharest, Romania;*
[3]*Toxicology Clinics, Floreasca Emergency Hospital, Bucharest, Romania*
[4]*Surgery Clinics, Sf. Pantelimon Emergency Hospital, Bucharest, Romania*
[5]*Research Institute of the University of Bucharest, ICUB, Romania*

ABSTRACT

Venous thromboembolism (VTE) is a disabling condition determined by thrombus formation in the venous system, which is preceded or followed by an inflammatory response of the blood vessel wall. VTE has a high incidence, is likely to be recurrent and can potentially be fatal. Diagnosis, treatment, and possible complications of lower limb deep vein thrombosis (DVT) are dependent on the anatomical location as well as on the extent of the process. One common complication of DVT is post-thrombotic syndrome (PTS), clinically characterized by chronic pain, enlarged veins, skin induration edema, and other signs of the affected limb, while, in severe cases, it can lead to venous ulcers. Venous thromboembolism has a wide array of risk factors, and several studies showed that the increasing disease prevalence is proportional to the risk factors. Diabetes and obesity have emerged as global health issues that are associated with a wide spectrum of disorders, including coronary artery disease, hypertension, stroke, and VTE. Within this line of thought, the current study aimed to investigate the prevalence of both diabetes and obesity in patients with VTE. A retrospective study (January 2013 - December 2015) was carried out by collecting data from medical documents available in Floreasca Emergency Hospital Bucharest, Romania. The patients diagnosed with deep vein thrombosis, on the basis of Doppler ultrasound, were divided into two age groups: group A (59 patients aged ≤ 50 years) and group B (130 patients aged > 50 years).

We found that the obesity rate was quite high in DVT patients belonging to both age groups; specifically, 40 (68%) of the young subjects were obese, whereas in the second age group consisting of elderly patients (> 50 years old), the rate of obesity reached 35.38%. The type 2 diabetes prevalence was quite elevated (34.62%) in elderly DVT patients and low (5.08%) in young DVT patients. In addition, lipid alterations were identified in 48.46% of the elderly patients and in 33.9% of the younger DVT subjects. Altogether, we found a positive association of diabetes, obesity and lipid alterations in elderly DVT patients but further studies are required in order to establish the mechanisms underlying these associations.

Keywords: deep vein thrombosis, diabetes, obesity

REZUMAT

Tromboembolismul venos (TEV) este o afecţiune debilitantă, determinată de formarea de trombi în sistemul venos, fiind precedată sau urmată de un răspuns inflamator la nivelul peretelui vasului de sânge. TEV are o incidenţă şi recurenţă crescută şi poate fi fatal. Diagnosticul, tratamentul şi posibilele complicaţii ale trombozei venoase profunde la nivelul membrelor inferioare (TVP) depind de localizarea anatomică, precum şi de amploarea procesului inflamator. O complicaţie obişnuită a TVP este sindromul post-trombotic, caracterizat clinic de durere cronică, edem şi alte semne la nivelul membrului afectat, precum şi de ulcere venoase in cazuri mai severe. TEV are o gamă largă de factori de risc, iar mai multe studii au arătat că prevalenţa bolii este direct proporţională cu prezenţa acestora. Diabetul şi obezitatea au apărut ca probleme globale de sănătate, fiind asociate cu un spectru larg de tulburări, inclusiv boala coronariană, hipertensiunea arterială, accidentul vascular cerebral şi TEV.

În cadrul acestui studiu am investigat prevalenţa diabetului zaharat şi a obezităţii la pacienţii cu TEV. Un studiu retrospectiv (ianuarie 2013 - decembrie 2015) a fost realizat prin colectarea datelor din documentele medicale disponibile la Spitalul de Urgenţă "Floreasca", Bucureşti, România. Pacienţii diagnosticaţi cu tromboză venoasă profundă pe baza analizei Doppler au fost împărţiţi în două grupe de vârstă: grupul A (59 pacienţi cu vârsta ≤ 50 ani) şi grupul B (130 pacienţi cu vârsta > 50 de ani).

Am constatat că rata obezităţii a fost destul de ridicată la pacienţii cu TVP aparţinând ambelor grupe de vârstă. Astfel, 40 (68%) dintre subiecţii tineri au fost obezi, în timp ce în al doilea grup de vârstă, alcătuit din pacienţi vârstnici (> 50 ani), rata obezităţii a ajuns la 35,38%. Prevalenţa diabetului de tip 2 a fost destul de ridicată (34,62%) la pacienţii vârstnici cu TVP şi redusă (5,08%) la pacienţii tineri cu TVP. În plus, modificări la nivelul profilului lipidic au fost identificate la 48,46% dintre pacienţii vârstnici şi la 33,9% dintre subiecţii tineri. În total, s-a identificat o asociere pozitivă între diabetul zaharat, obezitate şi alterarea profilului lipidic la pacienţii vârstnici, dar sunt necesare studii suplimentare pentru a stabili mecanismele care stau la baza acestor asociaţii.

**Corresponding author:* Georgeta Trucă, email: getatruca@yahoo.com

INTRODUCTION

Diabetes mellitus (DM) is a chronic disease with a global incidence and the World Health Organisation (WHO) predicts that the number of individuals with diabetes will reach 366 million by the year 2030. Diabetes appears when the body cannot produce enough or effectively use insulin, and is determined by a genetic predisposition coupled with environmental factors [1].

It is well known that DM is a risk factor for cardiovascular diseases (CVD). Indeed, individuals with type 2 diabetes mellitus (T2DM) exhibit a higher cardiovascular morbidity and mortality compared to non-diabetics [2]. Adult people with diabetes show rates of mortality due to heart disease and stroke from two to four times higher compared to healthy individuals [3]. Patients with diabetes usually aggregate other comorbidities, such as obesity, hypertension, and dyslipidemia which all contribute to an elevated risk of CVD [4]. The underlying mechanisms that lead to accelerated atherosclerosis in patients with diabetes and consequently an increased prevalence of CVD are not well understood. Diabetes represents a systemic and chronic condition defined by extended elevated glucose levels, causing the vascular endothelium to absorb unhealthy levels of glucose. Gradually, this pathology leads to permanent damage of blood vessels, followed by endothelial dysfunction, altered platelet activation, and elevated blood coagulability, thus leading to increased risk of thrombi formation [5].

Venous thromboembolism (VTE) encompasses two distinct clinical entities: deep venous thrombosis (DVT) or pulmonary embolism (PE) and occurs at an incidence of approximately 1 per 1,000 annually in adult populations [6]. Deep vein thrombosis (DVT) represents a serious condition that occurs when blood clots (thrombi) form in the deep veins of the body. DVD typically affects the deep veins of the legs or the deep veins of the pelvis . VTE is predominantly a disease of the elderly and has a relative higher frequency in males [6] . PE occurs if the clot becomes detached and travels to the pulmonary arteries, and can be fatal. Despite the progress in the diagnosis and treatment of DVT of the lower extremities, one in every three patients will suffer from post-thrombotic sequelae (PTS) which are severe in about 10% of the cases and harbor deep socio-economic consequences [7].

The prevalence of lipid alterations, diabetes and obesity in a Romanian patients cohort diagnosed with deep vein thrombosis was evaluated in this study.

MATERIALS AND METHODS

This is a retrospective study analyzing the period starting from January 2013 until December 2015. The data was collected from the medical documents available at Floreasca Emergency Hospital Bucharest, Romania. The method used in this study is observational and descriptive. The study group included patients diagnosed with deep vein thrombosis of the lower limbs based on Doppler ultrasound, hospitalized in various wards of the Emergency Hospital, such as internal medicine, orthopedics, cardiology and general surgery. The Doppler ultrasound determined the presence of chronic venous insufficiency, the type of venous thrombosis - deep or superficial and its location - proximal and distal.

The group of patients with deep vein thrombosis (DVT) was composed of 189 patients of whom 54 had superficial vein thrombosis (SVT). General data such as age, gender, origin, as well as clinical and paraclinical data were collected for each patient. Based on their age, patients were divided into two groups: group A (59 patients aged $\leq$ 50 years) and group B (130 patients aged > 50 years). The clinical data identified the presence of unilateral leg edema or the entire leg edema and the presence of peripheral trophic disorders (erythema, infiltration, skin induration, cellulitis and venous ulcers).

The anamnesis data was used to identify the presence of comorbidities and risk factors such as obesity, diabetes, and lipid alterations.

The group of patients with deep vein thrombosis (DVT) comprised 189 patients, of whom 54 (28.6%) had superficial vein thrombosis (SVT). The patients were divided into two groups: patients aged $\leq$ 50 years (31.22%) and patients aged > 50 years (68.78%). Applying ANOVA with a variable depending on age and an independent variable belonging to one of the two groups, we obtained as a result the average age of the patients of the first group as 37.71 years old with SD = 8.445, and for those of the second group as 68.50 with SD = 11,133. The difference between the two means is statistically significant ($p<0.001$). The first group included 23 (38.98%) women and 36 (61.02%) men and in the second 62 (47.69%) women and 68 (52.31%) men.

RESULTS

The group of patients with deep vein thrombosis (DVT) consisted of 189 patients, of whom 54 (28.6%) exhibited superficial vein thrombosis (SVT). Patients were divided into two groups: pa-

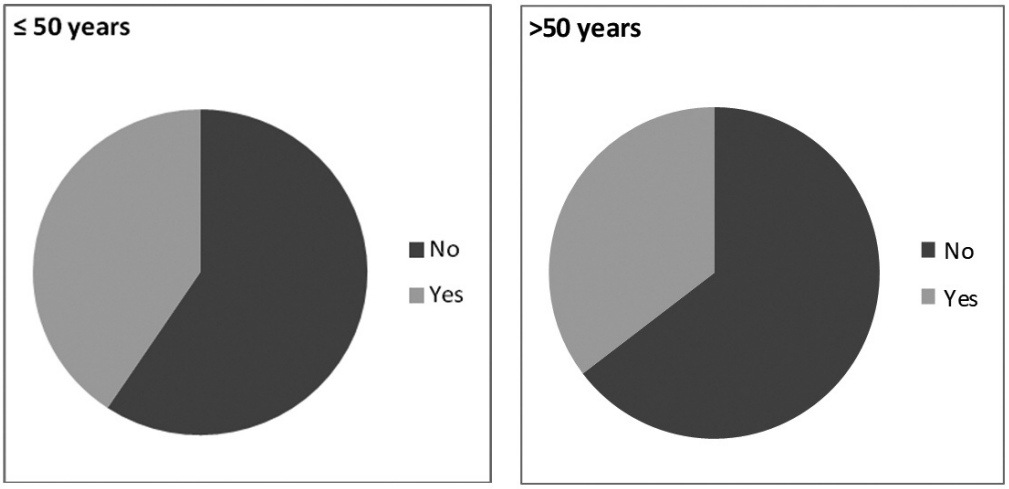

Fig. 1 - Prevalence of obesity in deep vein thrombosis patients divided based on their age (≤ 50 years and > 50 years old, respectively)

tients aged ≤ 50 years (31.22%) and patients aged > 50 years (68.78%). ANOVA analysis with a variable depending on age and an independent variable belonging to one of the two groups revealed that the average age of the patients of the first group was 37.71 years with SD = 8,445 whereas for the second group was 68.50 with SD = 11,133.

The first group consisted of 23 (38.98%) women and 36 (61.02%) men and the second one contained 62 (47.69%) women and 68 (52.31%) men. Our analysis showed that the prevalence of patients coming from urban areas was very high.

The first group of patients contained only 9 (15.25%) individuals coming from rural areas and 50 (84.75%) from urban areas.

The second group harbored 30 (23.08%) patients from rural areas whereas 100 (92%) patients were from urban areas.

Within the young (≤ 50 years old) DVT patients cohort, we identified a relatively high percentage of obesity. Indeed, 40.68% of the subjects were obese. In the second age group consisting of elderly patients (> 50 years old), the rate of obesity was 35.38% (Fig. 1).

Deep vein thrombosis has been linked with diabetes by several epidemiological studies [8]. Thus, the cohort of patients was also analyzed in terms of type 2 diabetes presence. We observed that the group harboring young patients had a low type 2 diabetes rate (5.08%). In contrast, the group consisting of elderly patients exhibited a type 2 diabetes rate of 34.62% (Fig. 2). This result is not surprising, considering the fact that type 2 diabetes usually develops in elderly individuals as a consequence of a chronic sedentary lifestyle and of unhealthy high-fat and high-sugar diets.

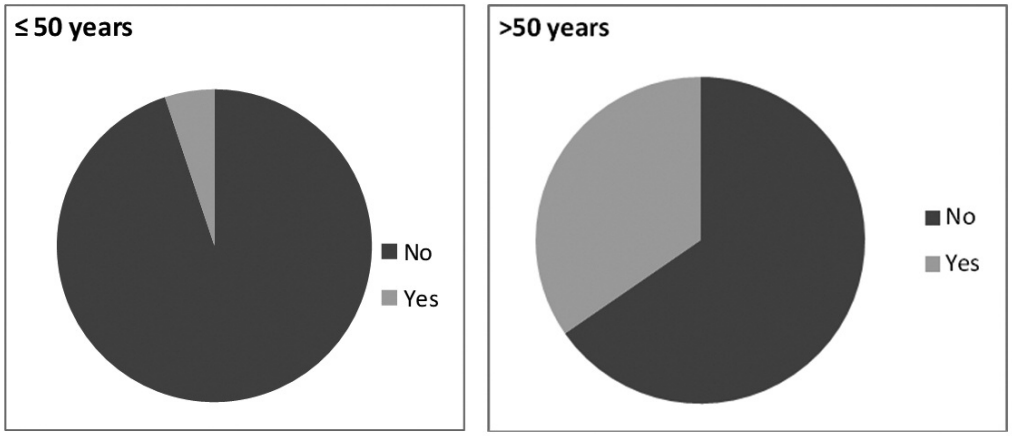

Fig. 2. Prevalence of type 2 diabetes in deep vein thrombosis patients divided based on their age (≤ 50 years and > 50 years old, respectively)

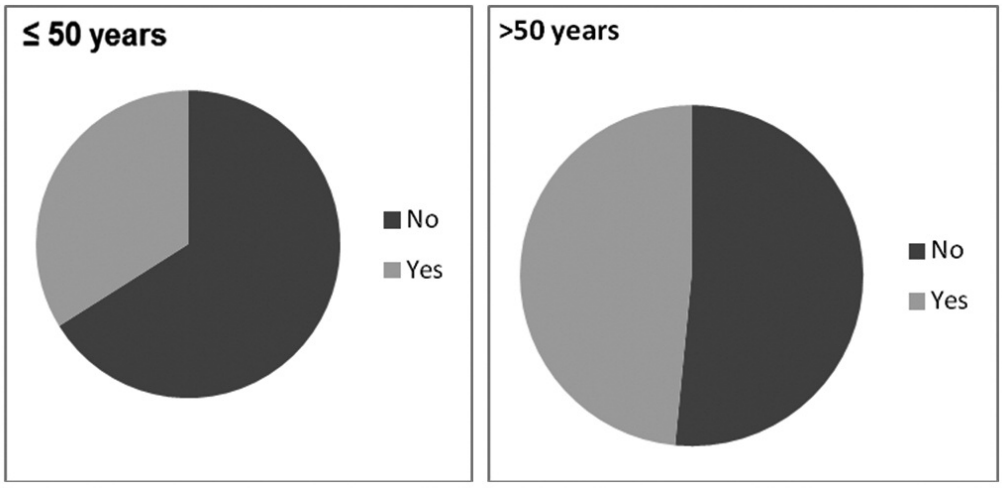

Fig. 3. The presence of lipid alterations in deep vein thrombosis patients divided based on their age (≤ 50 years and > 50 years old, respectively)

Lipid alterations including elevated levels of serum total cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-C), apolipoprotein B, and lipoprotein (a) were moderately present in young patients.

In terms of lipid alterations, patients in the ≤ 50 years study group revealed a percentage of 33.9%. A slightly higher (48.46%) level of lipid alterations was identified in elderly patients (Fig. 3).

DISCUSSION

Venous thromboembolism (VTE) appears as a result of the complex interplay between the genetic and environmental factors that influence the anticoagulant, coagulant, inflammatory procoagulant, and the fibrinolytic system, causing hypercoagulability and/or hypofibrinolysis. VTE is a multifactorial chronic disease that usually involves two or more risk factors.

VTE and atherosclerotic cardiovascular disease have in common many risk factors including hypertension, dyslipidemia, smoking, obesity and diabetes [9]. Studies linking diabetes to venous thromboembolism development showed conflicting results [8,10]. However, most of the epidemiological studies revealed an increased risk of deep vein thrombosis and pulmonary embolism among diabetics [11,12].

The elevated risk of venous thromboembolism associated with diabetes ranged from 40% in a large prospective US patient cohort [5] to 50% in a meta-analysis of more than 63,000 patients [11]. The risk of venous thromboembolism seems to be elevated in type 1 as well as in type 2 diabetic patients [12].

Elevated thrombin generation and an increased concentration of procoagulant cell-derived circulating microparticles in diabetic patients advocate that hypercoagulability may hold an important role in the increased frequency of venous thromboembolism [13]. Several studies have linked obesity with an elevated risk for VTE development.

Borch *et al.* showed that abdominal obesity was the only culprit for VTE development in a multivariable analysis that included risk factors such as a low level of high-density lipoprotein cholesterol (HDL-c), hypertension, impaired glucose metabolism, and hypertriglyceridemia [14].

In line with this, Steffen *et al.* showed that abdominal obesity was linked with idiopathic VTE after adjusting for age, race, smoking, triglycerides, HDL-c, glucose, and blood pressure [15]. Increasing body mass index BMI) above normal has been associated with a rising risk of VTE.

For instance, a prospective study involving 87,226 women showed that the relative risk of unprovoked PE that was not due to prior surgery, trauma, or cancer increased by about 8% per 1 kg/m^2 increase in BMI and reached a six fold higher risk among individuals with a BMI ≥ 35 kg/m^2 [16].

Altogether, in this study we found a positive association between diabetes, obesity and lipid alterations in elderly deep vein thrombosis patients, but further studies are required in order to establish the mechanisms underlying these associations.

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