Chapter 3

The Emerging Problems of Carbapenem-Resistant Gram-Negative Bacillary Pneumonia

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Additional information is available at the end of the chapter

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Abstract

Carbapenem-resistant Gram-negative organisms are increasingly isolated from lower respiratory tract infections. Limited treatment options are the main problems for physicians and clinical microbiologists who have to face such clinical cases. Bacteriological diagnosis, starting with accurate Gram smear performed from properly collected specimens and ending with antibiotic susceptibility testing, is essential. Morphological characters of bacterial cells provide important clues about the nature of infection, prior to bacterial isolation and identification. Attempts to find complementary options for the respiratory contamination and treatment of carbapenem-resistant Gram-negative bacillary pneumonia led us to test the susceptibility of 21 essential oils. Among them, *Thymus vulgaris*, *Eugenia caryophyllata*, *Origanum vulgare*, *Melaleuca alternifolia* and *Aniba rosaeodora* essential oils proved to be efficient against *Acinetobacter baumannii* carbapenem-resistant strain and *Escherichia coli* ATCC 25922. In an attempt to evaluate the magnitude of environmental spreading of the carbapenemase genes, 40 carbapenemase sequences of different organisms were compared. Carbapenemases show striking similarities inside each beta-lactamase class (A, D, and B), no matter their origin—environmental organisms or clinical isolates. Class B carbapenemases are most widely distributed, metallo-beta-lactamases being present in bacteria as well in Archaea.

Keywords: carbapenemase producer, nosocomial pneumonia, essential oils, environmental pollution

1. Introduction

Enterobacteriaceae and nonfermenters Gram-negative bacilli are increasingly isolated from lower respiratory tract infections. In patients in intensive care units (ICUs), the ventilator-associated pneumonia (VAP) is a constant concern for practitioners [1]. Improving the management of bacterial pneumonia led to guidelines for use by health-care personnel.

Nosocomial infections caused by Gram-negative bacteria resistant to carbapenems, mostly multiple-resistant, represent a challenge due to limited treatment options [2, 3]. Like other beta-lactamines, the carbapenems are included in preferred treatment regimens for various infectious diseases [4]. Antibiotic-resistant patterns of bacterial isolates from nosocomial infections are continuously changed. Therefore, it is of highest interest in finding alternative methods for preventing contamination with multidrug-resistant strains. Since ancient times, people have had an intuitive feeling that some spices have real benefits not only as air fresheners but also in that era they were the only therapeutic option for many diseases. Thus, they correctly guessed that concentrated lotions/oils are not only more efficient but easy to use and to store. Essential oils (EOs) from natural products proved their positive effects in various clinical conditions. Although the specific mechanisms of their components are not deciphered, many studies demonstrated certain biological activity of some phytochemicals. Terpinen-4-ol found in many EOs are demonstrated to act synergistically with chemotherapeutic agents in digestive malignancies [5]. There are many studies about antibacterial effects of EOs, mainly on supragingival plaque. The results are not constant, for example, some authors do not find a positive effect of tea tree oil on supragingival plaque [6].

Antibiotic-resistance genes are nowadays a constant presence not only in the hospital environment but are also more and more demonstrated in various ecosystems [7–9]. Antibiotic-resistance genes naturally already exist in organisms living in most diverse environments. Surely, antibiotic-resistance genes have an essential role in maintaining of inter-species equilibrium on specific ecosystems [10]. All living things from prokaryotes to eukaryotes are constantly exposed to a huge mixture of organic and inorganic compounds. Even if, nowadays, accurate methods exist to isolate and to characterize antibiotic-resistant microorganisms, it is not possible to calculate the influence of a myriad factors that interfere in every environment. An interesting study demonstrated the utility of transmission electron microscopy for observing of aquatic microorganism structural abnormalities in different environmental conditions [11]. The terrestrial ecosystem is also prone to be reshaped by human activities [12]. Intensive farming implies antibiotics, so the spread of intestinal bacteria which harbor antibiotic-resistance genes is an immediate consequence. This does not imply that once a certain bacteria species is present in a certain geographic area its antibiotic-resistance pattern remains unchanged. Atmospheric conditions, notably rainfalls, could contribute to spreading of contaminants from the soil to groundwater and greatly alter the count of microorganisms. Different bacterial species do not behave the same, antibiotic-resistance patterns differently changed, but certain beta-lactamines could be used as indicators of antibiotic resistance at least for *Escherichia coli* [13]. Consequently, it is not wrong to include antibiotic-resistance genes into the long list of environmental pollutants [14].

The physicians require bacteriological diagnosis on admission of the patients in ICUs and for surveillance of any nosocomial infection. For respiratory infections, Gram smears from sputum, endotracheal aspirate, or bronchoalveolar lavage are mandatory. Very often in ICUs, respiratory infections are due to carbapenem-resistant Gram-negative bacilli. In our opinion, testing volatile EOs, as complementary substances, for prevention of respiratory infections is not a futile idea. Finally, last but not least, wide use of antibiotics alters not only the hospital environment but also disturbs other ecological niches (water and soil). These topics will be our concern in the next sections.

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2. Carbapenem resistance

The carbapenems are sometimes the last-resort antibiotics for treating of extended spectrum beta-lactamase (ESBL) producing Gram-negative bacteria. But carbapenem resistance is increasingly reported in *Enterobacteriaceae* and nonfermenters like *Acinetobacter* spp. or *Pseudomonadaceae*. Carbapenem resistance is mainly due to carbapenemase (E.C. 3.5.2.6) production. Structural classification of beta-lactamases implies the primary structure (sequence homology) and distinguishes four molecular classes of beta-lactamases—A, C, D (serine beta-lactamases), and B (MBL-metallo beta-lactamase) [15–17]. Functional classifications of beta-lactamases are more closer to clinical issues [18, 19] and recognize three groups of beta-lactamases: Group 1 (Class C) cephalosporinases; Group 2 (Classes A and D) broad-spectrum beta-lactamases, inhibitor-resistant beta-lactamases, extended-spectrum beta-lactamases, serine carbapenemases, and Group 3 metallo-beta-lactamases. So far in this section, we have taken in consideration only different types of beta-lactamases as a resistance mechanism. Besides producing carbapenemases, the reduction of carbapenem influx into the periplasma is commonly observed in clinical isolates. Different mutations in porins significantly contribute to the failing of drug accumulation at appropriate concentration in periplasmic space; therefore, carbapenem resistance is often more complex than one thought at the first sight [20]. Readers, who wish to know further details regarding carbapenem resistance, should consult excellent papers devoted to this particular topic [21–24].

3. Microbiological investigations

Microbiological evaluation is mandatory for an adequate therapeutic regimen; accurate identification of the bacterial species is essential to avoid administration of broad-spectrum antibiotics. The optimal recovery of the pathogens ultimately depends on the accuracy of sample collection (sputum, endotracheal aspirate, or bronchoscopically obtained specimens by bronchoalveolar lavage). Bacteriological assessment of lower respiratory tract infections begins with care evaluation of Gram-stained smear performed from respiratory tract specimen. The low-power scanning provides a first sight of the quality of the sample—for sputum more than 10 squamous epithelial cells show oropharyngeal contamination. The examination with the oil immersion provides more details regarding bacterial morphology. The importance of this step in the management of bacterial pneumonia is well recognized. As illustrated in **Figures 1** and **2**, it is possible to anticipate the diagnosis toward a nonfermenter or an *Enterobacteriaceae*. *Acinetobacters* are short Gram-negative nonsporing bacilli but in exponential phase became coccoids often arranged in diplo. Many strains are encapsulated and sometimes retain the methyl violet in Gram's stain. The *Enterobacteriaceae* appear typically as Gram-negative nonsporing bacilli with parallel sides and rounded ends. There are wide ranges of derivatives from this classical appearance, from filamentous rods to coccoids. Some species are encapsulated, for example, *Klebsiella*, sometimes *E. coli.*

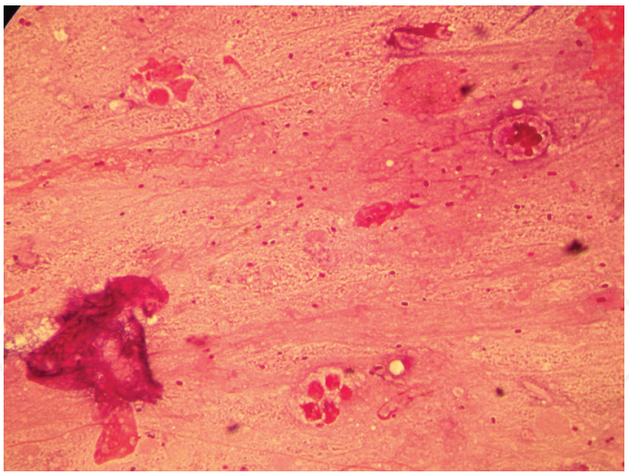

Figure 1. High-power examination (1000× magnification). Gram-stained smear of endotracheal aspirate shows Gram-negative coccobacilli isolated or in diplo (*Acinetobacter* spp.).

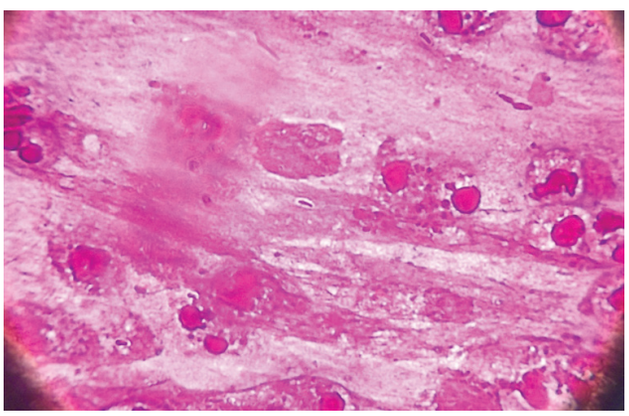

Figure 2. High-power examination (1000× magnification). Gram-stained smear of endotracheal aspirate shows Gram-negative capsulate rods.

Since this chapter is not intended to be a highly elaborate description of bacterial diagnosis of Gram-negative bacillary pneumonia, further details about isolation and bacterial identification were not be reviewed. However, the microscopic examination of clinical sample offers essential clues about the nature of bacterial infection. For busy clinicians—in ICUs the physicians have always needed a microbiologic response as quickly as possible—these details, provided in advance, could make the difference.

4. Therapeutic issues

4.1. Prevention of respiratory contamination

A particular issue of ventilator associated pneumonia (VAPs) is the risk of infection with multidrug-resistant strains and carbapenem-resistant bacilli too. Not surprisingly, severely

injured patients—VAPs and burn patients—are most prone to infection with carbapenem-resistant species. The chief question for carbapenem-resistant Gram-negative bacillary pneumonia is how to efficiently prevent them. First of all, could these infections be stopped? In the hospital environment, hand hygiene and alcohol-based disinfection remain, undeniably, the sanitation gold standard. Rigorous monitoring of patients at admission and an accurate history are early stages in identification of patients with documented multidrug-resistant strains for further isolation or, at least clustering separately to prevent the risk of cross-contamination [25, 26]. Respiratory contamination depends on so many circumstances, almost impossible to eliminate, that the specific strategies are designed in order to reduce VAP, rather than to eliminate such infections [27]. What else could be taken in consideration apart from already established strategies? As it was underlined in introduction, from the ancient times, people are aware of the so-called air purification performed intuitively by burning scented substances or widespread use of all sorts of perfumes, plant extracts, or spices.

4.2. Antibiotic therapy

Antibiotic regimens of carbapenem-resistant bacillary pneumonia often rely only on few antibiotics. Although there is not an ideal therapeutic regimen for the treatment of pneumonia due to carbapenem-resistant species, Polymixin B, Tigecycline, and Amikacine remain the most valid options [28]. A prerequisite for adequate treatment of VAPs is intravenous administration of the suitable antibiotic. Aerosolized antibiotics delivery has been experimentally studied in order to reduce the side effects of systemic administration of antibiotics. Efficiency of these methods relies on the antibiotics' ability on crossing the alveolar-capillary membrane [29]. An abundant literature is devoted to the issue of carbapenem-resistant strains. Because antibiotic resistance continuously evolved, clinical guidelines rapidly changed, therefore, a unique treatment scheme is almost impossible to establish. Clearly, we must look at the information provided by extensive epidemiological studies to up-date infection control and treatment options [30].

4.3. Inhibitory activity of essential oils

In spite of specific protocols implemented in ICUs, it is worthwhile to consider additional methods to prevent respiratory contamination. We should be considering the inhibitory effect of some essential oils (EOs), underlining the efficiency of volatile substances. EOs are more and more regarded as complementary to antibiotic therapy [31–35]. In our previous work, we demonstrated the high activity of EOs against *E. coli* for coriander (*Coriandrum sativum* L.), peppermint (*Mentha piperita* L.), and juniper (*Juniperus communis* L.) [36].

4.3.1. Materials and methods

We are interested in evaluating of the efficiency of some EOs against carbapenem-resistant *Acinetobacter baumannii* and *E. coli* ATCC 25922. *A. baumannii* is a ubiquitous nonfermenter species, found in soil, water, and clinical units, and *E. coli* is a constant presence of the normal microbiocenosis of humans and warm-blood animals. Of hundreds of natural products

commercially available, without prescription, only 21 volatile extracts (**Table 1**) were tested undiluted by two methods—diffusimetric method and aromatogram—described elsewhere [36]. Diffusimetric sensibility testing demonstrates the antibacterial activity by charging 5 mm diameter sterile paper disk with 2 or 5 μl of EOs. The aromatogram method is illustrated in **Figure 3**. Each experiment was performed three times at intervals of 2–3 days. The results are presented as mean and standard deviation (SD).

No	EO	Species	Family	Producer	Administration
1	Thyme	*Thymus vulgaris*	Lamiaceae	Fares	Internal use, aromatherapy, massage
2	Clove	*Eugenia cayophyllata*	Myrtaceae	Fares	Internal use, aromatherapy, massage
3	Eucalyptus	*Eucalyptus globulus*	Myrtaceae	Fares	Internal use, aromatherapy, massage
4	Juniper	*Juniperus communis*	Cupressaceae	Fares	Internal use, aromatherapy, massage
5	Lavander	*Lavandula angustifolia*	Lamiaceae	Fares	Internal use, aromatherapy, massage
6	Mint	*Mentha piperita*	Labiatae	Fares	Internal use, aromatherapy, massage
7	Pine	*Pinus silvestris*	Pinaceae	Fares	Internal use, aromatherapy, massage
8	Rosemary	*Rosmarinus officinalis*	Lamiaceae	Fares	Internal use, aromatherapy, massage
9	Tea tree	*Melaleuca alternifolia*	Myrtaceae	Fares	Internal use, aromatherapy, massage
10	Oregano	*Origanum vulgare*	Lamiaceae	Steaua Divina	Internal use, aromatherapy, massage
11	Negril	*Nigella sativa*	Ranunculaceae	Steaua Divina	Internal use, aromatherapy, massage
12	Lemon	*Citrus limonum*	Rutaceae	Steaua Divina	Internal use, aromatherapy, massage
13	Fennel	*Foeniculi fructus*	Apiaceae	Hofigal	Internal use
14	Sage	*Salvia officinalis*	Lamiaceae	Solaris	Aromatherapy, massage

No	EO	Species	Family	Producer	Administration
15	Sandalwood	*Santal amyris*	Rutaceae	Herbavit	Aromatherapy, massage
16	Seeds of apricots	*Prunus armeniaca*	Rosaceae	Herbavit	Internal use
17	Incense	*Boswellia serrata*	Burseraceae	Bionovativ	Internal use
18	Inhalant	*Pinus sylvestis, Salvia officinalis, Chamommilla recutita, Lavandula angustifolia* extracts, *Propolis cera,* Eucalyptus oil		Tisofit	Inhalations
19	Grapefruit	*Citrus paradisi*	Rutaceae	Solaris	Aromatherapy, massage
20	Orange	*Citrus sinensis*	Rutaceae	Solaris	Aromatherapy, massage
21	Rosewood	*Aniba rosaeodora*	Lauraceae	Solaris	Aromatherapy, massage

Table 1. The characteristics of the essential oils.

Figure 3. Aromatogram. In the middle of a sterile paper disk, with diameter of a Petri dish lid, 10 μl of essential oil was deposed. After sealing the assembly, the volatile effect was tested.

4.3.2. Results

4.3.2.1. Diffusimetric method

Figures 4–7 showing EOs inhibitory activity, demonstrate antibacterial activity for the two Gram-negative species tested. Before we comment on the implication of these results, it is worth considering the obvious antibacterial effect of some EOs. In particular, it is worth mentioning that we obtain more precise results—the smallest SDs—when 5 μl EOs are tested.

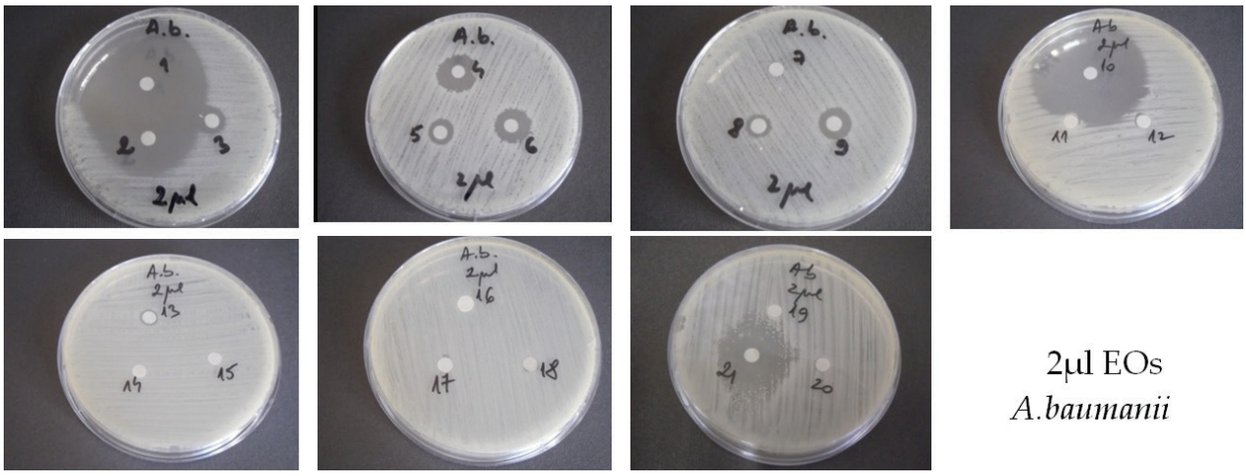

Figure 4. Diffusimetric method carbapenem-resistant *A. baumanii* tested by 2 µl EOs (see **Table 1** for the coresponding numbers).

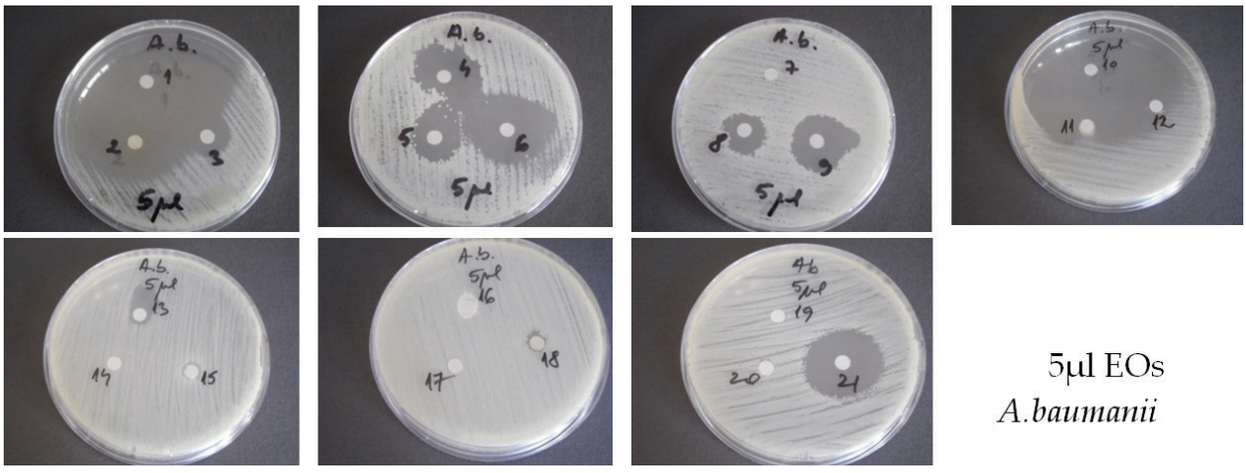

Figure 5. Diffusimetric method carbapenem-resistant *A. baumanii* tested by 5 µl EOs (see **Table 1** for the coresponding numbers).

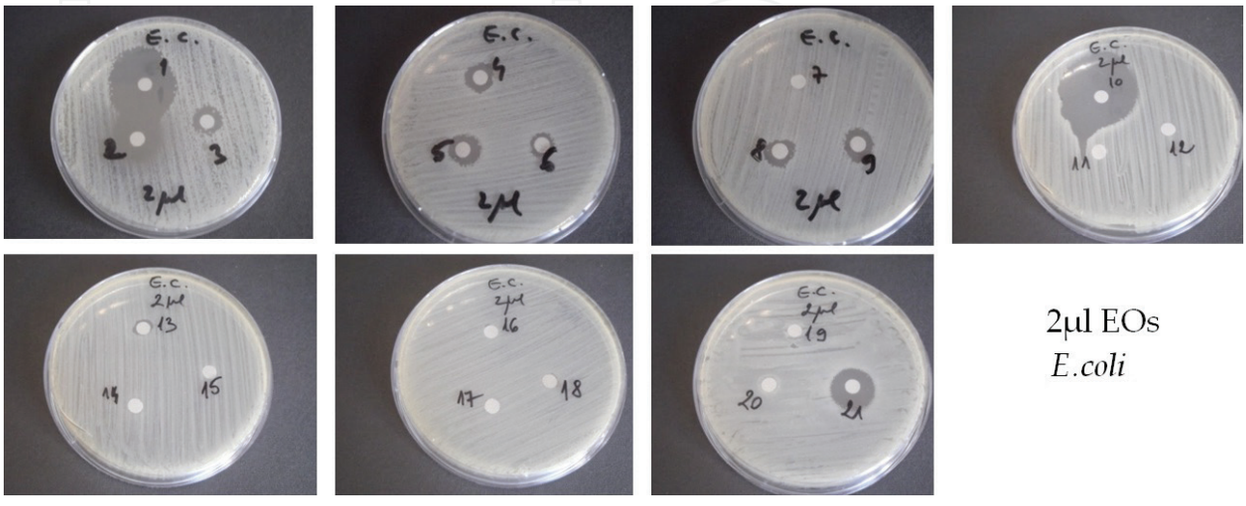

Figure 6. Diffusimetric method *E. coli* ATCC 25922 tested by 2 µl EOs (see **Table 1** for the coresponding numbers).

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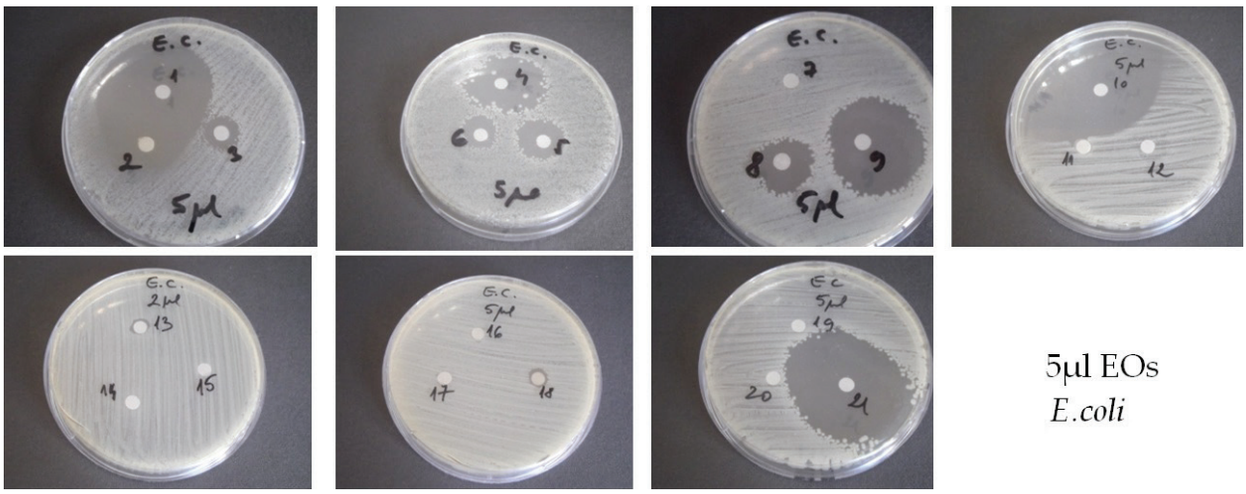

Figure 7. Diffusimetric method *E. coli* ATCC 25922 tested by 5 µl EOs (see **Table 1** for the coresponding numbers).

Thymus vulgaris, *Eugenia cayophyllata*, *Origanum vulgare*, and *Aniba rosaeodora* oils not only are by far the most effective but also obviously inhibit *A. baumannii* more efficiently than *E. coli*. Alternatively, *Melaleuca alternifolia* is slightly more effective against *E. coli* when diffusimetric method was used. Diffusimetric method also permits observing other important details. Although only clear inhibition zone was measured, *E. cayophyllata* shows residual growing, which is not measured and demonstrates a very good inhibitory activity. Also, it demonstrates an agonist effect in combination with *T. vulgaris*. Contrary, *O. vulgare* and *Nigella sativa* show an antagonistic effect.

4.3.2.2. Aromatogram

Aromatogram method (**Figures 8** and **9**) reveals more interesting evidence of usefulness of volatile effects of at least seven essential oils: *T. vulgaris*, *E. caryophyllata*, *O. vulgare*, *A. rosaeodora*, *Lavandula angustifolia*, *Mentha piperita*, and *M. alternifolia.* Surprisingly, some EOs, like mint, proved to be more effective when volatile effect is tested. The susceptibility testing was repeated after several weeks, but the results are inconsistent, mostly for mint, which showed no inhibitory effect after several weeks after opening the bottle. For other EOs (thyme, clove, eucalyptus, oregano, and rosewood) the antibacterial effect was not changed in time. Anyway, using single-use vials may be an option.

Synthetic results are listed in **Table 2**. At first glance, we noticed a remarkable antibacterial activity for *T. vulgaris*, *E. caryophyllata*, *O. vulgare*, and *A. rosaeodora*. Three other EOs could be considered as being efficient (*M. alternifolia*, *Lavandula angustifolia*, and *M. piperita*), but ten of them proved to have no antibacterial effect at any concentration tested. *Eucalyptus globulus* and *J. communis* could be considered for topical use, but not for their volatile properties. Four EOs (*E. globulus*, *Lavandula angustifolia*, *M. piperita*, and *Rosmarinus officinalis*) show multiple resistant colonies inside inhibition zone when *E. coli* was tested by aromatogram. Therefore, we consider them without antibacterial activity when volatile effect is considered.

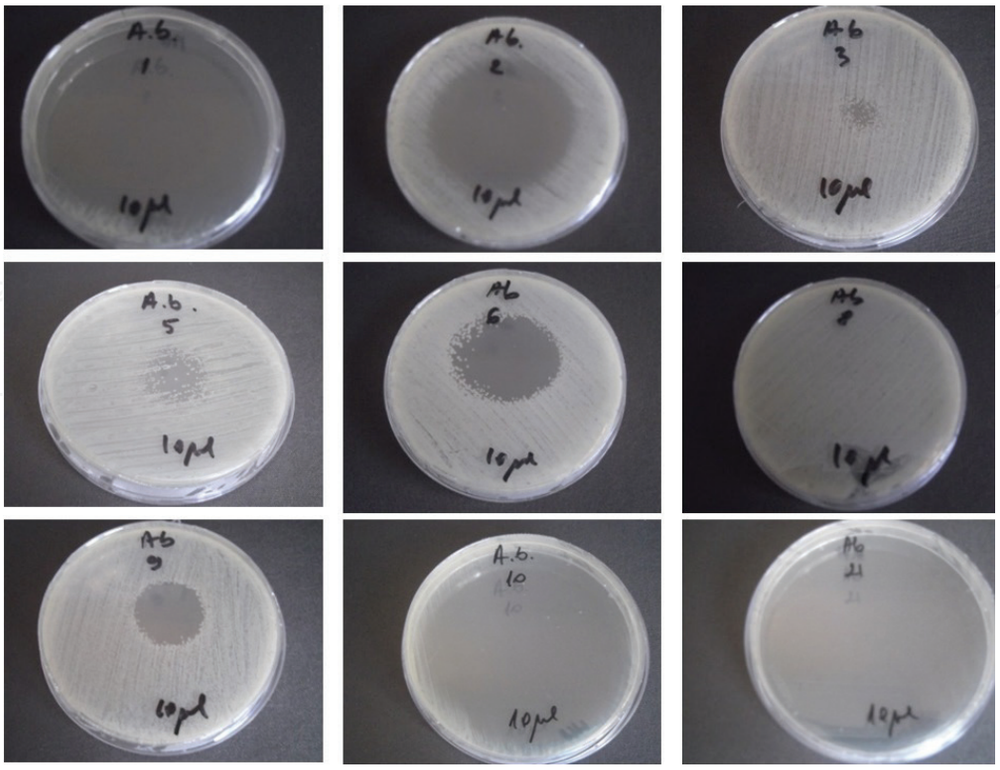

Figure 8. Aromatogram carbapenem-resistant *A. baumanii* tested by 10 µl EOs (see **Table 1** for the coresponding numbers).

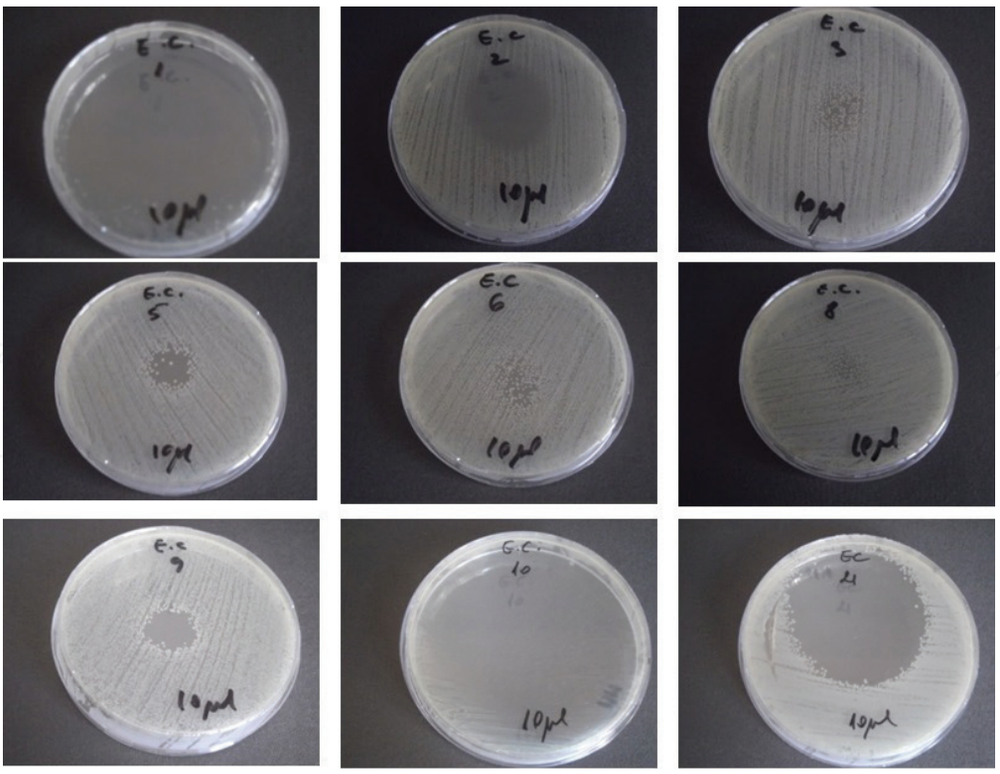

Figure 9. *E. coli* ATCC 25922 tested by 10 µl EOs (see **Table 1** for the coresponding numbers).

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EOs		Carbapenem-resistant *A. baumannii*			*E. coli* ATCC 25922		
		2 μl	**5 μl**	**10 μl**	**2 μl**	**5 μl**	**10 μl**
		Mean (SD)	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**	**Mean (SD)**
1	Thyme	38.33 mm (10.41)	66.67 mm (5.77)	70.00 mm (5.00)	28.00 mm (3.61)	65.00 mm (8.66)	**75.00 mm** (5.00)
2	Clove	21.67 mm (6.51)	32.33 mm (2.52)	46.67 mm (2.89)	19.00 mm (5.29)	29.33 mm (2.31)	**33.33 mm** (7.64)
3	Eucalyptus	9.67 mm (2.52)	16.00 mm (3.61)	NI	8.00 mm (1.73)	16.00 mm (3.46)	NI mrc
4	Juniper	16.33 mm (2.52)	23.00 mm (1.73)	NI	14.67 mm (4.62)	27.33 mm (2.52)	NI
5	Lavander	10.33 mm (0.58)	14.67 mm (2.52)	19.67 mm (5.51)	11.67 mm (0.58)	20.33 mm (3.51)	NI mcr
6	Mint	13.67 mm (2.31)	20.67 mm (6.51)	39.00 mm (6.56)	7.00 mm (0.00)	15.00 mm (1.00)	NI mcr
7	Pine	NI	NI	NI	NI	NI	NI
8	Rosemary	12.33 mm (±4.62)	16.33 mm (±1.53)	NI	10.33 mm (±3.51)	18.67 mm (±1.53)	NI mcr
9	Tea tree	15.67 mm (8.14)	24.67 mm (3.51)	26.33 mm (4.16)	18.33 mm (7.64)	23.33 mm (2.89)	11.00 mm (6.56)
10	Oregano	46.00 mm (±2.00)	70.00 mm (0.00)	77.67 mm (2.52)	29.67 mm (0.58)	40.33 mm (1.53)	76.00 mm (1.73)
11	Negril	NI	NI	NI	NI	NI	NI
12	Lemon	NI	NI	NI	NI	NI	NI
13	Fennel	6.00 mm (0.00)	6.33 mm (0.58)	NI	6.00 mm (0.00)	NI	NI
14	Sage	NI	NI	NI	NI	NI	NI
15	Sandalwood	NI	NI	NI	NI	NI	NI
16	Seeds of apricots	NI	NI	NI	NI	NI	NI
17	Incense	NI	NI	NI	NI	NI	NI
18	Inhalant	NI	NI	NI	NI	NI	NI
19	Grapefruit	NI	NI	NI	NI	NI	NI
20	Orange	NI	NI	NI	NI	NI	NI
21	Rosewood	16.00 mm (1.00)	32.33 mm (11.59)	80.00 mm (0.00)	14.67 mm (0.58)	30.33 mm (9.50)	50.00 mm (2.00)

NI—no inhibition; mrc—multiple resistant colonies.

Table 2. The inhibition of carbapenem-resistant *A. baumannii* and *E. coli* ATCC 25922 by 21 EOs. The results are reported as the mean (SD—standard deviation) of three independent experiments.

4.4. Discussion

In this study, commercial undiluted EOs were tested. There are some differences on viscosity, dispersion, vaporization, and other physical properties that, no doubt, influence the antibacterial activity. Although no proof can be given, the presence of antibiotic-resistance genes does not influence the efficiency of EOs. Therefore, an intuitive feeling turned our attention to the utility of these products in prevention or, why not, treatment of antibiotic-resistant Gram-negative bacillary respiratory infections. Although there are precise methods for identifying the chemicals with antibacterial activity, these are not important for our purpose. Note that here we are speaking of a screening of some commercial EOs that anyone can buy without medical prescription. Some authors demonstrate discrepancy of antimicrobial activity of EOs from different herbal varieties [37–40]. Of course, as we observed in our study, accurate description of physicochemical properties are needed [41], but for clinical purpose, the overall activity of EOs is relevant. In our opinion, a plant product should be considered as a distinct entity, and we can be certain that each component is synergistic to each other in a manner that exceeds the individual action of separated molecules. As we are speaking of living things, plant properties greatly depends on geographic area of collection, weather influence, manufacturing protocols, preservation conditions, species variety, and so on. Because natural plant products are considered safe, diverse possible applications were investigated: in agriculture for preventing crop diseases, monitoring soil characteristics [42, 43], or as industrial preservation solutions [44].

5. Environmental source of carbapenem-resistant strains

5.1. General considerations

Extensive studies are devoted to the ecotoxicity of industrial compounds or of pharmaceutical wastes [45]. Even though existing tools permit measurement of the concentration of any chemical in a certain geographic area, it is almost impossible to accurately estimate the influence of external factors that, no doubt, interfere with the spread or chemical transformation of any substance—like antibiotics. When biology occurs, the problems become more complicated. Nowadays, a different approach in follow-up to the intricate relationships of abundant microorganisms from a specific environment is needed. Antibiotics, like any other chemicals, do not differ in the way of spreading, accumulation, and changing certain environmental characteristics. It is not an exaggeration to state that multidrug-resistant microorganisms from hospital facilities are the nightmare of health practitioners. Analyzing bacterial species one by one provides certain information, but the big picture contains much more. Before starting to accumulate new data, there are huge unexplored resources, like public databases. Our concern was related to the magnitude of change on environmental microbiome by medical activities, especially the use of carbapenems. We extensively searched carbapenemases, or similar beta-lactamases, in protein databases available, and we compared their similitudes depending on their isolation source. The goal of searching similitudes between carbapenemase of clinical samples and their counterpart

of environmental origin was to assess the influence of medical activities in changing soil and water microbiota.

5.2. Data collection and methods

Herein, we have focused on comparison of the carbapenemases, from different sources, deposited in public databases—NCBI/National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/protein). The preliminary results were briefly presented in the First Conference of the Romanian Association of Laboratory Medicine [46]. Carbapenemase sampling was carried out to cover all beta-lactamase classes. In summary, 40 FASTA carbapenemase sequences were collected, and then a pairwise sequence alignment (EMBOSS Needle Program) and multiple sequence alignment (Clustal Omega tool) were performed [47–49]. The default settings were used. **Table 3** lists the characteristics of the sequences used for further comparisons. Sequences for Classes A, B, and D belonging to Archaea and bacteria were selected. Alignment sequence based on protein crystal structure was not possible due to lack of crystal structures available for beta-lactamases isolated from environmental samples.

5.3. Results and discussion

The major limit of the present study is the scarcity of carbapenemases of environmental source deposited in the public databases. Most of environmental beta-lactamases found belong to Classes B and D. The archaeal beta-lactamases found are metallo-hydrolase, and metal ions probably contribute to enzymes stability in extreme environmental conditions. Searching was extended to Eukaryota domain of life, but any sequences were included in the present study. Only two beta-lactamases-like of eukaryotic origin were retrieved—*Homo sapiens* (NP_057111) and *Caenorhabditis elegans* (NP_497107). Even they show MBL-folding, their similarity with other class B carbapenemases are very low, being more close to one hypothetical protein from *Sulfolobus acidocaldarius* (WP_01538554)—20.5% identity with NP_057111; 23.2% with NP_497107, respectively.

On the other hand, environmental Classes A, B, and D carbapenem-hydrolyzing enzymes are very similar with carbapenemases of clinical sources. Some carbapenemases, such as IMI-2, IMI-3, NDM-1, or OXA-48, are the same regardless their origin as observed by sequences pairwise comparison [50, 51]. For further statistical analysis, identity percent was recovered. Student's *t*-test assuming unequal sample variance was performed. The limit of this approach is due to the differences in the sequences length. The Needle program calculates identity, similarity, and score according to number of residues. Anyway, observing the data from **Table 4**, we noticed that environmental serine beta-lactamases are more similar with their clinical enterobacterial counterparts than with nonfermenters carbapenemases. The most striking differences are observed for subgroups IMI-3 of uncultured bacteria (ALJ52278) isolated from river sediment in China and OXA-48-like of uncultured bacteria (AJF40233) isolated from river sediment in Portugal. For ALJ52278 (IMI-3) beta-lactamase, 73.03% identity mean (CI 95 ± 25.33) with Class A carbapenemases of clinical *Enterobacteria* was noticed. More, sequences pairwise comparison reveals 93.50% identity with subgroups IMI-2 (*Enterobacter asburidae* WP_032491237), IMI-2 (*E. coli* AEU10762), and IMI-14 (*Enterobacter hormaechei*

Class	Source	Organism (accession number) isolation source	Beta-lactamase	Residues
Archaea				
MBL-fold	ES	*Sulfolobus acidocaldarius* (WP_011278945)	MBL fold metallo-hydrolase	305
		Sulfolobus acidocaldarius (WP_015385554)	Hypothetical protein	298
		Hadesarchaea archaeon (KUO42968)	MBL fold metallo-hydrolase	395
		Archaeoglobus fulgidus (WP_010877604)	MBL fold metallo-hydrolase	293
		Vulcanisaeta distributa (WP_013336995)	MBL fold metallo-hydrolase	306
Bacteria				
A	ES	*Enterobacter asburiae (*AIL29239)/water	IMI-2	192
		uncultured bacterium (ALJ52278)/river sediment, Haithe River, China	IMI-3	280
	E	*Escherichia coli* (WP_015059046)	GES-20	287
		Serratia fonticola (4EV4)	SFC-1 E166A mutant	283
		Serratia marcescens (1DY6)	SME-1	267
		Enterobacter asburiae (WP_032491237)	IMI-2	292
		Escherichia coli (AEU10762)	IMI-2	292
		Enterobacter hormaechei (APY16311)	IMI-14	292
	N	*Pseudomonas aeruginosa* (WP_021018677)	KPC	293
		Pseudomonas aeruginosa (4GNU)	GES-5	287
		Acinetobacter baumannii (3TSG)	GES-14	287
B	ES	*Serratia* sp. Sr_CGKV333_2014 (ALL98459)/ poultry flock soil, India	NDM_FIM-like_MBL-B1	270
		Acinetobacter junii (YP_009062718)/livestock farms, China	NDM-1 (plasmid)	270
		Acinetobacter calcoaceticus (YP_009060354)/ livestock farms, China	NDM-1 (plasmid)	270
	E	*Klebsiella pneumoniae* (4UWS)	VIM-26	266
		Klebsiella pneumoniae (5A87)	VIM-5	248
		Klebsiella pneumoniae (3SPU)	NMD-1	265
	N	*Pseudomonas aeruginosa* (4NQ2)	VIM-2	261
		Pseudomonas aeruginosa (AAR15341)	SPM-1	276
		Pseudomonas aeruginosa (ALU10771)	NMD-1	270
		Pseudomonas aeruginosa (AGH20684)	IMP-15	246
		Aeromonas hydrophila (1X8I)	CphA complex with Biapenem	227

Class	Source	Organism (accession number) isolation source	Beta-lactamase	Residues
D	ES	*Escherichia coli* (AJA05008)/*Halimione portulacoides*, Portugal	OXA-48 like	215
		Citrobacter freundii (AJA05009)/*Halimione portulacoides*, Portugal	OXA-48 like	190
		Pantoea eucalypti (AJA05006)/*Halimione portulacoides*, Portugal	OXA-181	173
		Uncultured bacterium (AJF40233)/river water, Portugal	OXA-48-like	248
	E	*Klebsiella pneumoniae* (5FAT)	Oxa-48 complex with Fpi-1602	243
		Klebsiella pneumoniae (4WMC)	Oxa-48 complex with Avibactam	242
		Escherichia coli (3QNC)	OXA-10	244
		Klebsiella pneumoniae (AGC60012)	OXA-244	265
	N	*Pseudomonas aeruginosa* (AAQ76282)	OXA-50	262
		Acinetobacter baumannii (ADB28891)	OXA-160	275
		Acinetobacter baumannii (4WM9)	OXA-24	245
		Acinetobacter radioresistens (ACE63186)	OXA-23	273
		Acinetobacter baylyi (ACH99101.1)	OXA-72	275

Table 3. The main characteristics of beta-lactamase sequences.

APY16311) (data not shown). The Class D carbapenemase AJF40233 (OXA-48-like) displays 80.35% identity mean (CI 95 ± 34.61) with its clinical *Enterobacteria* counterparts. Sequences pairwise comparison shows 91.00% identity with OXA-48-like (*Klebsiella pneumoniae* 5FAT and *K. pneumoniae* 4WMC) and 91.70% identity with OXA-244 (*K. pneumoniae* AGC60012) (data not shown). In contrast, for Class B carbapenemases, there are no notable differences when comparing environmental carbapenemases with those of clinical *Enterobacteria* or non-fermenters of Gram-negative bacilli origin. Particularly, all three NDM-1 carbapenemases of environmental sources included in the present study (**Table 3**) are identical with the carbapenemases isolated from *K. pneumoniae* (3SPU), *Enterobacteria*, or from the nonfermenter *Pseudomonas aeruginosa* (ALU10771). This is the most obvious illustration of long-term consequences of antibiotics use in livestock farms. Contrary, as it is shown in **Table 4**, Archaeal metallo-hydrolases show low similarities with other Class B carbapenemases.

Multiple sequence alignment of Classes A and D carbapenemases demonstrate that the active-site residues are very well conserved. Class B carbapenems, which mediate resistance to all beta-lactamases except aztreonam, are particularly interesting; they are found in bacteria, Archaea, and similar proteins, even in eukaryotes. Further, multiple sequence alignment (**Figure 10**)

Environmental samples	*Enterobacteria*	Nonfermenters	*P*-value
	Identity% Mean (CI 95)	Identity% Mean (CI 95)	
Class B			
Bacteria			
Serratia sp. (ALL98459)	51.07 (±70.37)	40.30 (±42.34)	0.65
A.junii (YP_009062718)	51.07 (±70.37)	40.30 (±42.34)	0.65
A. calcoaceticus (YP_009060354)	51.07 (±70.37)	40.30 (±42.34)	0.65
Archaea			
S. acidocaldarius (WP_011278945)	15.47 (±0.75)	15.56 (±3.04)	0.93
S. acidocaldarius (WP_015385554)	14.93 (±1.11)	14.48 (±2.88)	0.69
H. archaeon (KUO42968)	11.00 (±5.81)	13.18 (±2.57)	0.25
A. fulgidus (WP_010877604)	15.27 (±2.67)	15.38 (±1.59)	0.89
V. distributa (WP_013336995)	16.50 (±1.51)	16.60 (±2.21)	0.91
Class A			
E. asburiae (AIL29239)	54.87 (±17.00)	31.40 (±18.06)	0.02
Uncultured (ALJ52278)	73.03 (±26.33)	40.20 (±26.23)	0.03
Class D			
E. coli (AJA05008)	69.85 (±29.32)	28.54 (±2.37)	0.02
C. freundii (AJA05009.1)	67.78 (±25.43)	27.94 (±2.63)	0.01
Peucalypti (AJA05006)	60.93 (±21.63)	25.58 (±2.28)	0.01
Uncultured (AJF40233)	80.35 (±34.61)	32.26 (±3.02)	0.02

CI 95—Confidence Intervals 95%.

Table 4. Pairwise comparison of beta-lactamase sequences of environmental sources with *Enterobacteria* and non-fermenters carbapenemases.

highlights the notable differences between Archaea and bacteria. Residues involved in Zn^{2+} ions are very well conserved with the exception of Cys221, which is replaced by Asp in Archaea. However, nearby Cys residues were noticed. Since their crystal structures are not solved, we can just assume their involvement in metal ion binding.

The observation that Archaea contain different beta-lactamases demonstrates that human behavior has not profoundly altered natural environment. Or some microorganism communities have regulatory mechanisms so flexible that rapidly adapt at new environmental factors. For example, *Acinetobacter* is no longer considered just a free-living organism found in soil, water, and skin of human and warm-blooded animals, but an important multidrug-resistant pathogen.

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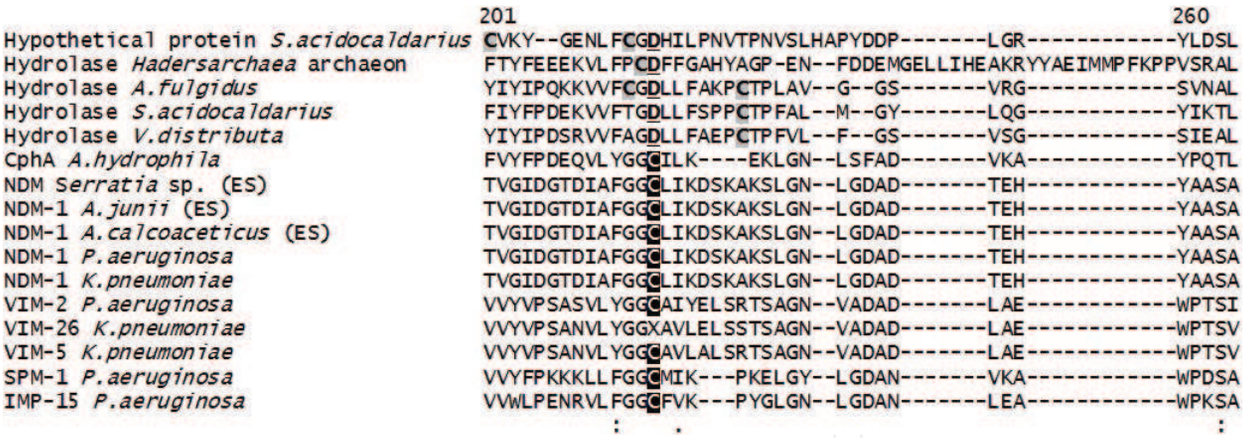

Figure 10. Multiple sequence alignment of Class B beta-lactamase. The cysteine residues involved in the second Zn^{2+} binding, highly conserved in bacteria, are written in white on black background; the residues noticed in Archaea are highlighted - the aspartic acid are underlined and the cysteine are written on grey background.

6. Final remarks

Carbapenems remain a valid option for the treatment of ESBL *Enterobacteriaceae* pneumonia. Prolonged treatments with beta-lactamine associated with other co-morbidities of the patients, rapidly changed the phenotypic pattern of resistance.

Essential oils have, no doubt, beneficial effects, but some providers excessively claim many possible effects—from antibacterial activity to neurological benefits—not always consistent with reality. Moreover, nowadays, there exists all sort of mixtures, and we can only hope that the ingredients are chosen following logical connections between their activities. Herein, these problems were not debated, but it is not a trivial question about the validity of all plant extracts. Just notice that the only mixture tested (an inhalant) proved no antibacterial activity, at least against *A. baumannii* and *E. coli* strains. On the other hand, tyme-clove association proved to be beneficial in Gram-negative bacilli respiratory infections. The oregano EO has an excellent antibacterial activity, but its effect is antagonized by negril EO. Other plant extracts could be added for their anti-inflammatory effect or just for changing the scent [52].

One important question arises from here—what else, apart from widely using of antibiotics, could influence the persistence of antibiotic genes in hospital facilities? Industrial wastes could remain for a long period of time in the environment, notably in groundwater [8]. Carbapenemases from hospital sources are, no doubt, the major factor in the evolution of these enzymes, hospital residues being, definitely, a source of wastewater pollution. Further, the carbapenemases produced by natural environmental bacteria and Archaea significantly contribute to selection of new mutations. Soil bacterial species greatly influences our life; therefore, a genome-scale metabolic network [53] has proved to be a valid approach to evaluate the complex dynamics of soil bacterial species, mostly in geographic areas near huge hospitals with many departments. Also, innate resistance to antibiotics has raised over time a growing interest for a rational design of new antibacterial compounds [54].

7. Conclusion

Carbapenem-resistant Gram-negative bacilli are one of the leading causes of nosocomial pneumonia. They are particularly involved in the outbreaks in ICUs. These strains are very often multidrug resistant, putting additional pressure on physicians and clinical microbiologists. Bacteriological diagnosis provides essential evidence for carbapenem-resistant Gram-negative bacillary pneumonia. From the very beginning, the Gram smear from respiratory specimens shows to be indispensable for an accurate diagnosis. Preventing respiratory infections in ICUs is a challenging issue; antibiotic prescription for any kind of acute respiratory tract infection does not benefit the patients. Besides, carbapenem-resistant bacilli already exist not only in clinical units but also alter environmental microbiota. It is time for a different approach in dealing with the antibiotic-resistance issues. An endless struggle with microorganisms does not work; these tiny creatures have incredible resources to deal with any new chemotherapeutic agent. We do not have the slightest idea of the long-term impact of widespread antibiotic use on environmental microorganisms. Careful analysis of existing data, like the evidence deposited in public databases, and reconsidering the antibacterial efficiency of natural products, such as EOs, could help at dealing with multidrug-resistant organisms.

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Conflicts of Interest

The author declare no conflict of interest. The funder had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, and in the decision to publish the results.

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