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Review

Bacterial Contamination of Flower Bee Pollen Production

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Abstract

The literature shows contradictory results regarding the prevalence of microorganisms, as well as the contamination of flower bee pollen. Here is a summary of the species-level differentiation of *Enterobacteriaceae, Pseudomonadaceae, Staphylococcaceae*, *Micrococcaceae* and *Bacillaceae* isolates obtained from investigations on fresh and dried flower bee pollen from different regions of Bulgaria.
***Key words**:* bee pollen, contamination, microorganisms

Резюме

Проучванията в научната литература за преобладаващите микроорганизми и контаминацията на цветния пчелен прашец показват противоречиви резултати. Представено е обобщение за видовото диференциране на изолати от сем. *Enterobacteriaceae, Pseudomonadaceae, Staphylococcaceae* и *Bacillaceae* при изследвания на неизсушен и изсушен цветен пчелен прашец от различни региони на България.

Introduction

Bee pollen is a valuable food collected by bees after they visit the flowers of plants. They gather it, adding specific enzymes to prepare the so-called pellets – small balls or conglomerates of pollen balls 2.5 to 3.5 mm of size (Central Cooperative Union, 1991). They are brought and stored in the cells of the beehive. Subsequently, bees additionally process the pollen in the cells, mixing it with nectar or honey and packing it with wax caps. Bee families use the pollen gathered in the hive as a main source of protein (Bogdanov, 2014). Under the popular name of 'perga' (from Russian) or bee bread, the pollen stored in cells, mixed with honey or wax, is not regulated for human consumption in the EC countries. Pollen is obtained under the form of pellets before its storage in cells. When bees pass through the openings of the pollen traps at the entrance of hives, the flower pollen stuck to their bodies falls on the ground and after purification, sieving, drying and packaging (Stratev *et al*., 2014) is allowed for human consumption (Ordinance No9, 2005). During the last years, consumers have turned their attention to natural products, with an increasing number of enthusiasts favouring bee pollen collected from pollen traps and dried.

At a global scale, general criteria for bee pollen quality and safety were proposed in 2008 (Campos *et al*., 2008). So far, there has been no detailed scientific investigation on the reliability of these criteria with respect to the quality and safety of this bee product. There are no certified specific methods for microbiological analysis of flower bee pollen consumed by humans. The literature data about microbial contamination of this product are also contradictory. Some researchers (Shevtsova *et al*., 2014) reported a high level of microbial contamination of bee pollen with bacteria from the family *Enterobacteriaceae* and it is acknowledged that some members of the family are human pathogens. It should be emphasised that the statutory documents stipulating the national requirements for bee honey (Central Cooperative Union, 1991; Ordinance No9, 2005) do not indicate any specific microbiological criteria and requirements for microbiological methods of examination of bee pollen.

The main food safety requirements comprise accurately formulated criteria for the presence of

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specific microorganisms. The inconsistent literature data about the microbial species diversity in bee pollen during its collection, processing and storage necessitate integral microbiological examinations.

The purpose of this research work was to present the summarised data about the microbial species from the families *Enterobacteriaceae, Pseudomonadaceae, Staphylococcaceae* and *Bacillaceae* isolated from dried and fresh bee pollen from 8 regions of Bulgaria, stored either frozen or in refrigerated conditions.

Materials and Methods

In June 2014, fresh and dried bee pollen samples were collected from eight regions of the country: Strandzha, Sliven, Stara Zagora, Shoumen, Lovech, Vratsa, Veliko Tarnovo and Karlovo. The samples originated from bee hives in industrial pollution-free areas, 3 km away from farmland with intensive crop production (Bogdanov, 2006).

In order to determine the microbiological parameters after one-year storage, pollen samples were vacuum-packed into polyethylene bags using a miniVac packaging machine (Vac-Star AG, Switzerland, available at: http://www.vac-star.com/en/p1-miniVAC.html).

Immediately before vacuum-packaging, the water activity of samples (Aw) was determined by automated analyser HygroLab (Rotronic AG, Switzerland). Measurements were run in duplicate and the result was retained after reaching a constant Aw value.

Until the time of microbiological analyses, samples of dried bee pollen were cold-stored (0-4ºC), (Ordinance No9, 2005), while fresh pollen samples were kept frozen at –18 °C (Dominguez-Valhondo *et al*., 2011).

Preparation of samples for microbiological analysis

Twenty-five g of bee pollen were diluted with 225 ml of buffered peptone water (Merck, Darmshtadt, Germany), then homogenised for 10 min at 200 rpm in a Stomacher and left for 30 min at room temperature. From this dilution, serial dilutions were made to 10-4 in sterile physiological saline.

Isolation of microorganisms from the Bacillaceae family

By means of an automated pipette and sterile pipette tips, aliquots of 100 µL from the initial and serial dilutions were spread onto plates with Plate Count Agar (PCA), (Merck, Darmstadt, Germany). Plates were incubated at 37°C and after 72 hours, isolates with similar colony and microscopic features were selected from the grown colonies for further species differentiation of *Bacillaceae* family members.

Determination of Enterobacteriaceae microbial counts and isolation of bacteria from the family Pseudomonadaceae

By means of an automated pipette and sterile pipette tips, aliquots of 100 µL from the initial and serial dilutions were spread onto plates with Violet Red Bile Glucose agar (VRBG Agar), (Merck, Darmstadt, Germany). Plates were incubated for 24 hours at 37 °C and then typical coliform colonies (those of dark-red colour and diameter ≥ 0.5 mm) were counted. The bacteria from the family *Pseudomonadaceae* were isolated from specific colonies grown on VRBD Agar (Merck, Darmstadt, Germany).

Isolation of bacteria from the family Enterobacteriaceae

Aliquots of 100 µl from the initial and serial dilutions were inoculated on MacConkey agar and XLD (Xylose Lysine Deoxycholate) agar (Merck, Darmstadt, Germany), and plates were incubated at 37°C for 24 h. The remaining amount of the initial dilution (1:10) was left for enrichment at 37°C for 18 h. Then followed a secondary enrichment in two enrichment broths: selenite broth (37°C, 24 h) and Rappaport-Vassiliadis medium (41°C, 24 h), (Merck, Darmstadt, Germany). By the 42nd hour, enrichment broths were inoculated on MacConkey agar and XLD agar (Merck, Darmstadt, Germany), and plates were incubated again at 37°C for 24 h.

If lactose-negative or lactose-positive *Enterobacteriaceae* colonies were detected after the 24-h primary incubation or after the 66-h incubation from enrichment broths on MacConkey and XLD agar plates, isolates were further analysed by Gram staining of microscopic preparations followed by the principal protocol for initial laboratory differentiation of *Enterobacteriaceae* on Kligler iron agar, motility test medium, indole and H_2S (Merck, Darmstadt, Germany), as well as *Salmonella* (Sifin Service GmbH, Berliner Allee 317-321, Berlin, Germany, http://sifin.de/) (Dinkov, 2016).

Isolation of bacteria from the Staphylococcaceae family

For identification of microorganisms from the family *Staphylococcaceae,* a preliminary enrichment of 1 mL of the initial dilution was made in 9 mL TSB supplemented with 7.5% sodium chloride. The latter was added as it suppresses other bacteria, helps the isolation of staphylococci and especially of enterotoxin-producing *S. aureus*, which is mark-

edly resistant to 7.5% NaCl (Koch, 1942). For selective enrichment of staphylococci, a specific Giolotty and Cantoni Broth (GC) (Merck, Darmshtadt, Germany) was also used.

The enrichment broths incubated at 37ºC for 24 h were re-inoculated on Baird Parkar Agar (BPA), (Merck, Darmstadt, Germany) supplemented with 0.0025% potassium tellurite (Sawhney, 1986). The plates were incubated at 37°C and after 24-48 h, the typical dark-black staphylococcal colonies were observed. The subsequent investigation of isolates was done by Gram staining, determination of catalase and oxidase activity, inoculation of BP agar sectors for single colony growth. After re-incubation (24-48 h, 37°C), the obtained pure cultures were examined again by Gram staining, catalase and oxidase activity, presence of pigmentation after inoculation on ordinary agar, haemolytic activity on blood agar, and plasma coagulase activity on rabbit plasma.

The subsequent identification to the species level was done with 9 isolates from each region with similar colony and primary biochemical features, tentatively identified as members of the families *Enterobacteriaceae, Pseudomonadaceae, Staphylococcaceae* and *Bacillaceae*. They were stored until analysis at -18ºC in Eppendorf tubes with TSB (Tryprone Soy Broth), (Merck, Darmstadt, Germany) supplemented with 20% glycerol. Prior to identification of species, isolates were cultured on blood agar for growth of single colonies of pure cultures, followed by 24-hour incubation at 37°C. The species differentiation of obtained pure cultures was done through the identification system BioLog Gen III microplates (BioLog, Hayward, USA).

Identification of isolates through the system BioLog Gen III microplates (Biolog, Hayward, USA)

In brief, separate colonies from the grown isolates were taken with a special swab with a pointed tip, put into tubes containing IF-A medium and homogenised to obtain microbial suspension for GEN III plates. In each well of the GEN III plate, 100 µL microbial suspension was added and plates were incubated at 33°C for 24 h. Results were read visually by the change of colour in the wells and compared to positive (10^{th} well) and negative (1^{st} well) controls. Data were interpreted with OmniLog software of BioLog Gen III microplate system (Biolog, Hayward, USA).

Results

Table 1 presents the results from water activity and total *Enterobacteriaceae* bacterial counts determination. The highest water activity was established for dried bee pollen from the Strandzha (0.450/21.9ºC) and Lovech (0.388/23.8ºC) regions, followed by Sliven (0.309/23.2ºC), Veliko Tarnovo (0.298/22.3ºC) and Shoumen (0.183/20.2ºC) regions. Dried bee pollen samples had water activity between 0.183 and 0.450, and fresh pollen samples – within 0.715 and 0.725 (Table 1).

The highest *Enterobacteriaceae* bacterial counts were established in dried bee pollen from Strandzha (8.5×10^3 CFU/g), followed by Shoumen (3.6×10^3 CFU/g), Sliven (1.5×10^3 CFU/g), Veliko Tarnovo (1.4×10^3 CFU/g) and Lovech (7.5×10^2 CFU/g) regions. Microorganisms from the family *Enterobacteriaceae* were not established in the

Table 1. Water activity and total count of microorganisms from *Enterobacteriaceae* family in flower bee pollen from different regions of Bulgaria

Regions	Dried bee pollen		Regions	Fresh bee pollen	
	Water activity (Aw / °C)	Enteroba-cteriaceae (CFU/g)		Water activity (Aw / °C)	Enteroba-cteriaceae (CFU/g)
Lovech	0.388/23.8ºC	7.5×10^2	Vratsa	0.715/20.8ºC	1.32×10^4
Strandzha	0.450/21.9ºC	8.5×10^3	Strandzha	0.718/22.2ºC	5×10^4
Shoumen	0.183/20.2 ºC	3.6×10^3	Shoumen	0.725/20.6ºC	1.4×10^4
Sliven	0.309/23.2 ºC	1.5×10^3	Sliven	0.722/23.4 ºC	3.7×10^4
V. Tarnovo	0.298/22.3 ºC	1.4×10^3	-		
St. Zagora	0.234/22.9ºC	not detected *	-		
Karlovo	0.403 /25ºC	not detected *	-		

* not detected - *no microorganisms from the family Enterobacteriaceae were present following direct inoculation of 100 mL of the initial dilution on VRBD agar*

Table 2. Microorganisms from *Enterobacteriaceae and Pseudomonadaceae* families in fresh and dried flower bee pollen from different regions of Bulgaria.

Isolated species	Dried bee pollen	Fresh bee pollen
	Regions	Regions
Pantoea species		
Pantoea agglomerans	All regions without Shoumen	Vratsa and Sliven
Pantoea agglomerans bgp 6	Shoumen	Shoumen and Strandzha
Citrobacter species		
Citrobacter freundii	Lovech, Shoumen, St. Zagora	-
Proteus species		
Proteus vulgaris	Sliven	Shoumen and Strandzha
Proteus mirabilis	Shoumen	Shoumen
Serratia species		
Serratia odorifera	V. Tarnovo	-
Serratia liquefaciens/ grimesii	-	Strandzha
E. coli species		
E.coli	-	Vratsa and Shoumen
Pseudomonas species		
Flavimonas oryzihabitans	-	Vratsa

samples from Stara Zagora and Karlovo regions. The highest total microbial counts for *Enterobacteriaceae* in fresh pollen samples were found again in the samples from Strandzha (5×10^4 CFU/g), Sliven (3.7×10^4 CFU/g), Shoumen (1.4×10^4 CFU/g), and Vratsa (1.32×10^4 CFU/g) regions.

Table 2 presents the data about *Enterobacteriaceae* and *Pseudomonadaceae* species detected in bee pollen. The analyses have shown mostly coliforms and some other *Enterobacteriaceae* members, which have not been so far reported in this bee product.

In all studied regions, microorganisms from the *Pantoea* spp. were present in both dried and fresh bee pollen. In the latter, *Pantoea agglomerans* was detected in Vratsa and Sliven regions. *Pantoea agglomerans bgp 6* was established only in dried and fresh bee pollen samples collected form the Shoumen regions, as well as in fresh product from Strandzha. *Citrobacter freundii* was found in dried pollen samples from Lovech, Shoumen and Stara Zagora regions (Table 2).

Dried bee pollen from the Sliven region was shown to contain *P. vulgaris*. This microbial species was also found in fresh pollen samples from Strandzha and Shoumen regions. *P. mirabilis* was present only in both types of bee pollen from Shoumen.

Bacteria from the genus *Serratia* were demonstrated only in bee pollen from two of the regions. Dried pollen samples from Veliko Tarnovo contained *Serratia odorifera*, and fresh pollen from Strandzha – *Serratia liquefaciens/grimesii*.

The fresh pollen samples from two of the surveyed regions (Vratsa and Shoumen) were positive for *E.coli*. Bacteria of the *Pseudomonadaceae* family were only found in fresh bee pollen from the Vratsa region - contamination with *Flavimonas oryzihabitans* was detected (Table 2).

Table 3 gives the results about detected *Staphylococcaceae* microbial species. Our examinations did not establish coagulase-positive staphylococci, acknowledged as human pathogens. Dried bee pollen from all studied regions except for Strandzha contained *S. hominis ss hominis*. This organism was present only in fresh pollen samples from the Sliven region.

Dried and fresh pollen samples were most

Table 3. Microorganisms from *Staphylococcaceae* family in fresh and dried flower bee pollen from different regions of Bulgaria

Dried bee pollen		Fresh bee pollen	
Staphylococcaceae			
Regions	Microorganisms	Regions	Microorganisms
Shoumen	*S. hominis ss hominis*	Shoumen	*S. epidermidis*
Strandzha	*S. epidermidis*	Strandzha	*S. epidermidis*
Sliven	*S. hominis ss hominis*	Sliven	*S. hominis ss hominis*
Stara Zagora	*S. hominis ss hominis*	Vratsa	*S. epidermidis*
Karlovo	*S. hominis ss hominis*	-	
V.Tarnovo	*S. hominis ss hominis*	-	
Lovech	*S. hominis ss hominis*	-	

Table 4. Microorganisms from *Bacillaceae* and *Micrococcaceae* families in fresh and dried flower bee pollen from different regions of Bulgaria

Dried bee pollen		Fresh bee pollen	
Fam. Bacillaceae			
Regions	Microorganisms	Regions	Microorganisms
Shoumen	*B. pumilis* *Arthrobacter globiformis*	Shoumen	*B.subtilis* *B. pumilis* *A. globiformis*
Strandzha	*B. pumilis*	Strandzha	*B. pumilis*
Sliven	*B. pumilis*	Sliven	*B. pumilis*
Stara Zagora	*B. amyloliquefaciens* *B. pumilis*	Vratsa	*B.subtilis* *A. globiformis*
Karlovo	*B. amyloliquefaciens* *B. pumilis*	-	
V.Tarnovo	*B. pumilis*	-	
Lovech	*B. pumilis* *B. amyloliquefaciens*	-	

commonly contaminated with *B. pumilis* (Table 4). While *B. subtilis* was not found in dried pollen samples, it was detected in fresh product from two of the studied regions – Shoumen and Vratsa.

The second most prevalent species in fresh bee pollen after *B. pumilis* was *B.subtilis*, which was not found in any of the dried samples. In our studies, the microbial species *B. amyloliquefaciens* in dried bee pollen samples (Stara Zagora, Karlovo, Lovech regions) and *A. globiformis* in the fresh pollen from Vratsa and Shoumen were demonstrated for the first time (Table 4).

Discussion

It is acknowledged that higher water activity values are beneficial for the development of microorganisms. This is an important factor guaranteeing food safety during production and subsequent storage (Rahman, 2010). The high A_w is proved to induce the development of microorganisms (Mathlouthi, 2001), including pathogenic bacteria which replicate extensively at water activity values > 0.85, while molds could develop at water activity values >0.6 (Rahman, 2010).

The water activity values substantially lower than 0.85 detected in dried bee pollen in our studies suggest that when drying was properly done, there should be no preconditions for development of microbial pathogens in this product (Table 1). This is confirmed by the established highest levels of water activity (0.450/21.9ºC), corresponding to the highest total microbial *Enterobacteriaceae* counts in dried pollen samples from Strandzha (8.5×10^3CFU/g). Higher *Enterobacteriaceae* microbial counts proportionally to higher water activity levels (0.718/22.2ºC) were found again in fresh pollen samples from the Strandzha region (5×10^4 CFU/g) (Table 1).

At the same time it should be noted that the water activity in fresh pollen from all regions was >0.6 (Table 1), which according to Rahman (2010) is a precondiion for development of some microorganisms in stored bee pollen. The relatively low water activity in some samples, in which detected *Enterobacteriaceae* counts were higher (Table 1), could be attributed to the secondary contamination of pollen between the moment of its harvesting from bees, its transportation to the hive and the subsequent primary processing (Gilliam, 1979; Serra and Escola, 1997).

Coliforms are Gram-negative, oxidase-negative, non-spore forming, aerobe and facultative anaerobe rods. Although not taxonomically distinct, the group is functionally defined as lactose-fermenting, gas and acid producing bacteria at 35ºC. The group includes, apart from the genus *Escherichia* (*E. coli* in particular*),* the genera *Citrobacter, Enterobacter* and *Klebsiella*. Some authors place in this group *Serratia* and *Hafnia*. Coliforms are normal residents of the intestinal tract of animals and men, and are also encountered in the environment, soil and water. About 1% of coliforms, mainly *E. coli*, are detected in animal and human gastrointestinal tract. The family *Enterobacteriaceae* comprises about 20 genera including coliforms, as well as some other foodborne microorganisms proven to be pathogenic, for example the members of *Salmonella, Shigella* and *Yersinia* genera (Tortorello, 2003).

The data about the lack of bacteria from the family *Enterobacteriaceae* in dried bee pollen indicate the essential role of production hygiene and timely drying as the main factors impeding the development of enterobacteria (Table 1).

According to reported projects for international standards for bee pollen, no more than 100 *Enterobacteriaceae* CFU/g are recommended in this product (Campos *et al*., 2008). Compared to these recommendations, samples of vacuum packed dried bee pollen cold-stored for 1 year exhibited values from 7.5×10^2 – 8.5×10^3 CFU/g (Table 1). The interpretation of these data should take into consideration the fact that apart from coliforms, the *Enterobacteriaceae* family also includes other bacteria, some of them (*Salmonella*, *E. coli* etc.) pathogenic, as well as ubiquitous opportunistic or facultatively pathogenic microorganisms, which rarely cause disease in humans (Sanders and Sanders, 1997). Yet, there are no data for contemporary evaluation of the risk from the presence as well as about criteria for the admissibility of opportunistic or facultatively pathogenic microorganisms in foods with respect to consumer safety.

On the other hand, scientific studies indicate that some diseases in plants could be transmitted through pollen (Card *et al.,* 2007; Flores *et al.,* 2005). Envionrmental pollution, the activities of bees during pollinaiton of plants, collection and transportation of pollen, human activities during pollen collection from pollen traps and its primary processing (sieving, drying, packaging) are all important factors for contamination of pollen, as well as the air at the site where plants grow (Gilliam, 1979; Serra and Escola, 1997). Other environmental factors – rain, dew, fog, spray irrigation, could be also involved in the contamination of pollen (Lacey and West, 2007). It is acknowledged that prior to and during bringing the pollen into the hive, bees moisten the pollen with nectar and place it in the baskets on their legs, which makes the product susceptible to additional microbial contamination.

Our results for increased counts of *Enterobacteriaceae* in fresh bee pollen (1.32×10^4 - 5×10^4 CFU/g) point at the primary role of secondary contamination with these bacteria, which could occur following their presence in the environment, on plants, bees, and during harvesting of pollen from pollen traps or during its sieving or drying. The data indicating lower enterobacterial counts in dried pollen ($7.5\text{x}10^2$ – $8.5\text{x}10^3$ CFU/g) confirm the importance of proper drying of the product with respect to the inhibition of microbial development and maintenance of acceptable level of safety for consumers.

It is acknowledged that *P. agglomerans* which was prevalent in bee pollen according to our studies (Table 2), is used in agriculture as a biological antagonist of fungal diseases in plants (Nunes *et al*., 2001). Some authors believe that *P. agglomerans* was detected in bees and in bee products in hives after the visit of bees on plants (Loncaric *et al*., 2009). *P. agglomerans* has been also isolated from various plants in the Black Sea region (Mudryk, 2012). Recently, some researchers have classified *P. agglomerans* as opportunistic pathogens, which are dangerous mainly for immunocompromised subjects. The bacterium was detected in patients with arthritis (Kratz *et al*., 2003), as well as occasionally as a causative agent of septicaemia in newborns (Bergman *et al*., 2007). It was found out that *Pantoea* spp. rarely causes disease in healthy people (Sanders and Sanders, 1997). *P. agglomerans* is not included in the recommendations for European microbiological criteria to bee pollen (Campos *et al*. 2008). On the basis of our results, we suggest

future examination of bee pollen for contamination with *P. agglomerans* in our geographical regions as well, which could justify the inclusion of this microorganism in the microbiological requirements to the product.

The additional investigations of antibiotic sensitivity of five strains of *P. agglomerans* and *P.agglomerans bgp 6* isolated from dried and fresh bee pollen in 4 of the surveyed regions (Shoumen, Strandzha, Sliven, Karlovo) with regard to their sensitivity to antibiotics from the main groups of antibacterial drugs used in human medicine: ß-lactams (amoxicillin + clavulanic acid: 20/10 μg), aminoglycosides (gentamicin), amphenicols (chloramphenicol), tetracyclines (doxycycline), quinolones (enrofloxacin) and cephalosporins (cephalotin) allowed to conclude that there was a minor risk for transfer of antibiotic resistance through *P. agglomerans* in bee pollen (Dinkov, 2016).

Members of the genus *Citrobacter* (*Citrobacter diversus*) have been encountered in bee pollen (Belhadj *et al*., 2014). So far, there are no data about the occurrence of *Citrobacter freundii*, which was detected in dried bee pollen samples from the region of Lovech, Shoumen and Stara Zagora (Table 2). It should be noted that bacteria from genera *Citrobacter* and *Pantoea* spp. do not pose a risk to healthy people and are frequently encountered in the environments. They are also placed in the opportunistic species group, causing neonatal meningitis and abscesses in humans (Joaquin *et al.,* 1991).

Proteus vulgaris is another opportunistic or facultatively pathogenic microorganism, causing disease in subjects with immunodeficiency disorders, kidney fibrosis or HIV (Steinkamp *et al*., 2005). It is demonstrated that when predisposing factors are present, *Prot. vulgaris* could induce urinary tract, skin and wound infections (Berg *et al.,* 2005). There is evidence that *P. mirabilis* has been more commonly found in the intestinal content of diarrhoeic subjects than in healthy persons, which could be attributed to its role as a human intestinal pathogen (Müller, 1986). The interpretation of our results should take into consideration the fact that the role of *P. mirabilis* as a human pathogen is not entirely elucidated, which could be dangerous after consumption of bee pollen, as well as its absence in recommendations for microbiological criteria to bee pollen (Campos *et al*., 2008). Last but not least, it should be outlined that *Prot. mirabilis* was detected only in fresh and dried pollen samples from a single region (Shoumen, Table 2).

The microorganism *Serratia liquefaciens/grimesii* (Table 2) is also classified as a potential human pathogen and is encountered in several plants (Berg *et al*., 2005). The available literature sources provide no data about the involvement of *Serratia odorifera*, detected in the dried bee pollen from Veliko Tarnovo, in human diseases.

E. coli were detected in fresh bee pollen from the Vratsa and Shoumen regions (Table 2). Furthermore, the organism was not detected in bee pollen from Shoumen after drying and one-year storage in vacuum packages. Drying is recommended as the first step of the primary processing of floral bee pollen with regard to inhibition of *E. coli* replication. This microorganism was not found in dried pollen samples, in line with recommendations stipulating its absence in dried pollen intended for human consumption (Campos *et al*., 2008).

Flavimonas oryzihabitans, found in fresh bee pollen from the Vratsa region (Table 2) was initially detected in rice, hence its name (Kodama *et al*., 1985). So far, there is no information about the occurrence of this bacterium in bee pollen. *Pseudomonas* spp., which is also from the group of opportunistic bacteria, could cause mainly skin and wound infections (Berg *et al*., 2005). Some authors reported *Flavimonas oryzihabitans* as an agent of postoperative septicaemic infections in newborn babies (Freney *et al*., 1988) and of peritonitis secondary to peritoneal dialysis (Bending *et al*., 1989).

Future research should investigate the possible relationship between skin infections occurring from the collection of fresh bee pollen from pollen traps contaminated with opportunistic bacteria from the family *Enterobacteriaceae* (Table 2). There is therefore a need for observation of a higher level of precautions not only during processing, but also using disposable gloves when working with pollen traps and during the primary processing of the product.

S. hominis ss hominis is a member of the resident microflora of human skin, occasionally causing infections in immunocompromised people (Palazzo *et al*., 2008). Gram-positive cocci and especially *S. epidermidis* are encountered in bees and bee pollen (Gilliam and Lorenz, 1983). It has also been evidenced that *S. epidermidis* as a part of normal skin microflora rarely causes disease, except for immunosuppressed patients (Levinson, 2010).

The wide spread of *S. hominis ss hominis* in dried bee pollen proved in our studies after its being primarily processed (Table 3), suggest a possible secondary contamination with this bacterium during sieving and drying. The opposite relationship

was found in *S. epidermidis*. It has been detected in most of the surveyed regions, but was present in dried pollen samples only from the Strandzha region (Table 3). The absence of *S. epidermidis* could be attributed to the mechanical removal of the agent with the secondary contaminants of pollen during sieving.

Bacillus sp. were isolated from 59% of samples stored in cells of honeycombs (bee bread) and from only 18% of samples collected from bees outside the cells. *B. megaterium* is the most commonly encountered species. *B. circulans* and *B. alvei* were detected only in pollen from honeycomb cells, but not in stored food (Gilliam *et al*., 1990).

Some isolates of family *Bacillaceae* detected during our studies were identified as *B. subtilis* (Table 4), determined by other researchers as a common species in both collected pollen and pollen stored in comb cells. Other representatives of this family, isolated from bee pollen, are *B. megaterium*, *B. licheniformis*, *B. pumilus* and *B. circulans* (Gilliam, 1979).

It is demonstrated that some *B. cereus* and *B. pumilis* strains could produce enterotoxins and therefore could be considered dangerous in cold stored foods due to their psychrotrophic nature and potential of growth at temperatures ≤ 6ºC (Ray and Bhunia, 2014). It should be also noted that of the bacilli acknowledged as human pathogens, some references determine *B. cereus* as surely pathogenic. Allowances of up to 50 CFU/g of this bacterium in powdered milk intended for children until 6 months of age are already regulated (Regulation 1441, 2007). This *Bacillaceae* member was detected in none of the regions surveyed during our study (Table 4).

B. subtilis is used for plant disease control (Idris *et al*., 2004). In our studies, the share of *B. subtilis* among *Bacillaceae* isolates from fresh bee pollen was considerable (Table 4). It should be emphasised that *B. subtilis* was not encountered in dried bee pollen. This could be due to sieving which removes the particles carrying additional *B.subtilis* contamination from the environment. On the basis of results about absence of *B. subtilis* in dried pollen samples (Table 4), we could hypothesise that sieving, proposed as an important element of the primary processing of pollen (Stratev *et al*., 2014) has minimised the chance of contamination.

The predominant member of family *Bacillaceae* in our studies was *B. pumilis* (Table 4). This bacterium is psychrotrophic, able to replicate at the low temperatures at which the product was usually stored in our experiments. The less frequent detection of *B. pumilis* in fresh pollen could be attributed to its storage in a frozen state (-18 ºC).

Bacillus amyloliquefaciens (Table 4) was also associated with its occurrence in plants. It stimulates plant growth and is used for control of bacterial and fungal plant diseases. That is why some authors consider the microorganism as an alternative for plant disease control (Borriss *et al*., 2011).

It has been shown that *B. pumilus* and *B. subtilis* are the main representatives of the family *Bacillaceae*, encountered in spices (Muhamad *et al*., 1986). *B. pumilus* was also encountered in cold-stored flours (Rogers, 1978). The pathogenic potential of this bacterium and the possibility for production and accumulation of endotoxin posing a risk to people is still unclear. With this regard it should be noted that *Bacillus* spp. do not replicate at $A_w < 0.92$ (EFSA, 2005). To prevent the development of *B. cereus,* the storage of foods at < 4ºC is recommended as at these temperatures the spores of *B. cereus* could not develop into vegetative forms and hence, accumulate toxin (EFSA, 2005).

It should be outlined that dried pollen samples in our study exhibited water activity between 0.183 and 0.450, whereas fresh pollen samples: from 0.715 to 0.725 (Table 1). Therefore, the one-year cold storage of dried or frozen storage of fresh pollen did not create prerequisites for *Bacillus* spp. replication.

Soil microorganisms from *Arthrobacter* spp. are found in bees and wax moths (Gilliam and Lorenz, 1983). Some authors use *A. globiformis* for testing the antibacterial peptides in the haemolymph of bees for evaluation of their immunity level (Korner and Schmid-Hempel, 2004; Sadd and Schmid-Hempel, 2009). In our studies, *A. globiformis* was detected in pollen samples from Vratsa and Shoumen regions (Table 4).

Having investigated the antibacterial activity of bee pollen (bee bread) obtained from bee-processed product stored in honeycomb cells, Baltrušaytite *et al*. (2007) concluded that it was due to flavonoids and phenolic acids contained in pollen, also reported in other research on this product (Carpes *et al*., 2009).

The occurrence of *Staphylococcus* spp. and *Bacillus* spp. in pollen (Tables 3 and 4) showed that the observed antibacterial effect of pollen against them could be exhibited only by high-concentration ethanol solutions (40-90%) (Carpes *et al*., 2007), but not by consumption of floral bee pollen not processed in ethanol. In support of this, no antibacte-

rial activity of ethanol extracts (0.02%-2.5% v/v) of *Laurus nobilis* L. pollen against *S. aureus*, *B. cereus* and *B. subtilis* was found out (Erkmen and Özcan, 2008).

Gamma irradiation at a decontamination dose of 1.0 Mrad is recommended after processing a drying laboratory evidence of contamination of bee pollen with bacteria from the family *Bacillaceae*, which was successfully applied for reduction of the content of spore-forming bacteria up to 10^3 CFU/g, required by Japanese Hygienic Standard for spice (Muhamad *et al*., 1986).

Conclusion

In dried flower bee pollen from 8 regions of Bulgaria after one-year vacuum-packed cold storage (0-4ºC) microorganisms that could cause enteric diseases in humans were not established (Regulation 1441, 2007).

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Review

On the Action of N^1, N^1-Anhydro-bis(β-hydroxyethyl)-Biguanide Hydrochloride (Abitylguanide, Moroxidine, ABOB) Versus RNA Viruses

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Abstract

The review considers the compound N^1, N^1-anhydro-bis(β-hydroxyethyl)-biguanide hydrochloride (abitylguanide, ABOB) as the first applied in the clinical practice anti-flu drug, which later did not prove efficacious, was officially rejected and classified as an agent whose merit only deserved a place in the history of antivirals. The compound manifested some activity against other RNA-containing viruses (paramyxo) but with contradictory effects, strongly dependent on the test methods used and on the virus inoculation dose (multiplicity of infection).

Key words: abitylguanide, influenza viruses, paramyxoviruses

Резюме

Обзорът разглежда съединението N^1, N^1-anhydro-bis(β-hydroxyethyl)-biguanide hydrochloride (abitylguanide, ABOB), първият приложен в лечебната практика антигрипен препарат, който по-късно не доказа своята ефикасност, бе официлно отхвърлен и класифициран като средство, имащо място само в историята на антивиралите. Съединението показа известна активност спрямо други РНК вируси (парамиксо), но с противоречиви ефекти, силно зависими от приложения тест метод и от вирусната инокулационна доза (множественост на инфекцията).

Concerning the anti-influenza action

In 1960, Bo Melander announced that the compound designated by the generic name abitylguanide (moroxidine) or by the abbreviation ABOB, manifested a protective effect against influenza pneumonia in mice, infected via inhaling influenza viruses A/Puerto Rico/8 (H1N1) and B/Lee, expressed by a reduction of the degree of both pathologic changes and virus titer in the lungs. This marked the start of intensive research on the anti-influenza activity of this substance and of the biguanide derivatives in general during a period when antiviral chemotherapy was making its first steps. It was established that ABOB possessed some activity on the replication of influenza viruses A(H1N1), A(H2N2) and B *in vitro* (Rada, 1962), *in ovo* (Liu and Engle,1960; Goret and Pilet, 1963) and *in vivo* (Melander, 1960a; Goret and Pilet, 1963).

In spite of its moderate antiviral effect in experimental conditions (according to contemporary criteria), in the absence at that time of other anti-influenza substances and proved to be absolutely harmless (Söberg, 1960a; Cutting, 1962), ABOB comparatively quickly was included in clinical trials in influenza infections, including via the double-blind study scheme, organized by the Swedish company AB/KABI (Stockholm). In those trials the compound was applied in tableted combination with small doses of methatropine nitrate and methscopolamine nitrate, named Flumidin (AB/KABI). By the end of 1961, Flumidin [Virustat (Delagrange, France), Virugon, Virunil, Virusmin, Spenitol, Influmin (Polfa, Poland) and other trade names] was tested on as many as 15,000 persons with influenza A(H1N1), A(H2N2) and B, demonstrating, according to the judgment of a series of researchers (Söberg, 1960b; Almberg, 1960; Melander, 1960b, 1963; Jordan, 1961; Sandring, 1962; Zetterberg *et al.*, 1962; Wheatley, 1963; Haglind, 1964; Kitamoto, 1964; Klettenhammer, 1964), a generally favorable, although moderate, effect at prophylactic administration (in tablets of 300-500 mg twice daily), evidenced by a certain decrease in the incidence of influenza. When applied in a ther-

apeutic dose of 300-500 mg thrice daily for 7-14 days starting on the first days of the symptomatic appearance, a mild course of the disease was observed (Söberg, 1960a,b; Kitamoto, 1964; Haglind, 1964; Klettenhammer, 1964). In the meantime, reports appeared emphasizing the value of Flumidin as a prophylactic means, which called into question its therapeutic effect (Söberg, 1960a, b; Dumon *et al.*, 1963; Haglind, 1964), while other reports completely rejected its efficacy (Parker *et al.,* 1962; Jackson, 1963; Stanley *et al.,* 1965).

Whereas Parker *et al.* (1962) considered Flumidin use in viral infections of the upper respiratory tract of unspecified etiology, the double-blind studies carried out by Jackson (1963) and Stanley *et al.* (1965) merited serious attention. The completely negative results of these very well controlled trials proving the inefficacy of ABOB in influenza gave a grounding of the Standing Joint Committee on the Classification of Proprietary Preparations of Great Britain to place this medicament in the category of unacceptable drugs, as a drug with improved value (1967) (Bauer, 1972). Despite the data about a certain prophylactic effect of ABOB combined with homatropin-methylbromide and vitamin C (Morgalin, Chinoin, Hungary) in influenza infection in a closed children group (Ambró and Nagy, 1974), monographies and publications on antiviral substances after 1970 passed over in silence this substance or pointed it as an example of non-effective anti-influenza drug (Bauer, 1972; Sidwell and Witkovski, 1979; Zlydnikov *et al.,* 1979). Zlydnikov *et al.* (1979) classified ABOB within the pathogenetic and symptomatic action in influenza.

The analysis of the results of experimental *in vitro* and *in vivo* studies carried out with ABOB, estimated according to the contemporary criteria for antiviral activity, demonstrates that the involvement of this compound in clinical trials (and even in *in vivo* tests) for influenza has been groundless.

We have carried out tests to compare the effect of ABOB and other anti-influenza antivirals – rimantadine hydrochloride, ribavirin and mopyridone on the model of the orthomyxovirus - influenza virus A/chicken/Germany/27 (FPV, Weybridge) (H7N7) by the plaque-reduction method and demonstrated that ABOB did not possess a marked inhibitory effect (Galabov *et al*., 1981).

Effect towards paramyxoviruses and other RNA viruses

In 1964, we established that ABOB tested against the two antigenic variants of paramayxovirus type 1 Sendai, Kuroya strain (Japanese variant) and 960 strain (Vladivostok variant), at non-toxic concentrations (375 – 1100 μg/ml) manifested some inhibitory effect on the replication of this virus in calf kidney primary cell cultures. On the basis of its value, this effect could be classified as a moderate to a weak one, being strongly dependent on the multiplicity of infection (m.o.i.). A study on the mode of action of the compound emphasized the role of suppression of viral protein synthesis (characterized via the direct immunofluorescent method) (Galabov, 1966, 1968a, 1968b). ABOB manifested a marked suppression effect on the virus growth in embryonated hen eggs (Galabov, 1966).

In the meantime, Rada and Závada (1962), using their own developed agar-diffusion plaque-inhibition method, reported that ABOB was ineffective against another paramyxovirus, Newcastle disease virus. In contrast, Gherganov *et al.* (1986) established a pronounced antiviral effect of the compound against strain P of this virus, using a novel *in vitro* test system, tracheal organ cultures of pheasants. A complete protection of the ciliary activity (at 150-1500 50% cilliostatic doses) under ABOB was registered.

ABOB was inactive against togavirus Western equine encephalomyelitis virus in the agar-diffusion plaque-inhibition test (Rada and Závada, 1962), against poliovirus 1 (the Sabin's LSc-2ab vaccinal strain and the virulent Mahoney strain) (Galabov, 1978) in one-step growth cycle experimental design in HeLa (S_3 clone) and KB cells, respectively, and against vesicular stomatitis virus replication in L cells (Galabov, 1978). Some inhibitory effect was registered against bovine leukemia virus reproduction in FLK cells, but not against Mc-29 avian leukemia virus (Galabova and Galabov, 1983).

In conclusion,

the biguanide ABOB activity on the replication of RNA-containing viruses could be considered as a weak one, with a contradictory effectivity, the selectivity ratio values being markedly below the requirements of the established contemporary methodological criteria for antiviral substances. The pioneer role of this substance as an anti-flu agent did not meet the requirements of the anti-flu experimental chemotherapy, when the compound was studied by contemporary methodical tools. Therefore, ABOB only remained as an ineffective anti-influenza drug in the history of this research area.

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Review

Multidrug-Resistant Gram-Negative Bacteria – a Problem for Hospital Infectious Pathology

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Abstract

The spread of multidrug-resistant /MDR/ Gram-negative bacteria in the hospital setting is a world-wide problem. In this study we present data about the resistance to antimicrobials of some problematic for hospital infectious pathology bacteria – *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Acinetobacter baumannii* and *Pseudomonas aeruginosa* on the model of a multiprofile hospital. There has been an increase in *ESBLs*-producing *E. coli* and *K. pneumoniae* strains, isolated especially in some so-called "risky units" at the Military Medical Academy /MMA/ for the last years. There were also registered the first strains *E. coli,* producing metallo-beta-lactamase NDM1. The data show specific association between the *blaNDM-1* and *rmtB* genes conferring high-level resistance to all aminoglycosides in these strains. Non-fermenting *A. baumannii* and *P. aeruginosa* strains isolated usually are multiresistant with high-level resistance to carbapenems and other beta-lactams and quinolones, and the resistance to carbapenems in *A. baumanni* strains is associated with the production of *Oxa 23*, *Oxa 58* and *Oxa 72* carbapenemases, but not metallo-beta-lactamases.

Keywords: Gram-negative bacteria, resistance to antimicrobials, ESBLs, carbapenemases

Резюме

Разпространението на множествено-резистентни Грам-отрицателни бактерии в болничната среда е световен проблем. В това проучване представяме данни за резистентността към антимикробни лекарствени средства на някои проблемни за болничната инфекциозна патология микроорганизми - *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Acinetobacter baumannii* и *Pseudomonas aeruginosa* на модел многопрофилна болница. Наблюдава се нарастване на щамовете *E. coli* и *K. pneumoniae*, продуциращи широко-спектърни бета-лактамази /ESBL/, изолирани предимно в т.н. „отделения с повишен риск за развитие на инфекция" във Военно-медицинска академия / ВМА/ през последните години. Регистрирани са също първите щамове *E. coli,* продуциращи металобеталакмаза *NDM-1*. Данните показват специфична асоциация межд *blaNDM-1* and *rmtB* гените, кодиращи високо ниво на резистентност към аминогликозиди в тези щамове. Неферментиращите глюкозата Грам-отрицателни бактерии *A. baumannii* и *P. aeruginosa* се изолират обикновено като множествено резистентни с високо ниво на резистентност към карбапенеми и др. бета-лактамни антибиотици, хинолонови производни, като резистентността към карбапенеми при щамовете *A. baumanni* се свързва с продукцията на *Oxa 23*, *Oxa 58* и *Oxa 72* карбапенемази, но не и с метало-бета-лактамази.

Introduction

Many studies were focused on the significance of the multidrug-resistant Gram-negative bacteria as a cause of severe bacterial nosocomial infections worldwide, with two thirds of the 25 000 annual deaths occurring only in Europe /www.medscape.com/ viewarticle/717606-3/. Initial reports of *ESBLs*-producers in the eighties and early nineties essentially focused on the descriptions of nosocomial outbreaks, mainly in intensive care unit settings, caused by *Klebsiella* and *Enterobacter* spp. which produced various *TEM*- or *SHV- ESBL* subtypes. More recently, novel *ESBLs* coding genes (e.g. of the *CTX-M* family) derived from plasmid mobilization of environmental or soil organisms

have emerged worldwide in several enterobacteria – *Enterobacter* species, particularly in *E. coli.* (Schoevaerdts *et al*., 2011). Since 2000, there has been a rapid increase in *Enterobacteriaceae* members (*E. coli, Klebsiella pneumonia*) and some carbapenemase-producing nonfermenting Gram-negative bacteria (*Acinetobacter baumannii, Pseudomonas aeruginosa*). According to the CDC, the proportion of *Enterobacteriaceae* that were resistant to carbapenems increased from 0% in 2001 to 1.4% in 2010, with most of the increase recorded in *Klebsiella spp* (CDCVital signs, 2013). Of particular importance for the resistance to carbapenems it is also the registered co-production of multiple beta-lactamases resulting in clinical resistance to all classes of beta-lactams (Bush, 2013).

The aim of this study is to present data on the resistance and multi-resistance of some problematic for hospital infectious pathology Gram-negative bacteria on the model of a multiprofile hospital.

Background

Among all of the bacterial resistance problems, gram-negative pathogens are particularly worrisome, because they are becoming resistant to nearly all drugs that would be considered for treatment. The most serious, life-threatening infections are caused by a group of drug-resistant bacteria that the Infectious Diseases Society of America (IDSA) has labeled the „ESKAPE" pathogens, because they effectively escape the effects of antibacterial drugs. /http://www.medscape.com/. Additionally, in 2013 the CDC categorized the 18 microorganisms that pose the greatest antimicrobial resistance threats to public health into 3 threat groups: urgent, serious, and concerning (Solomon, 2013; CDC, 2013). The pathogens assigned to the urgent and serious categories require more monitoring and prevention activities. The most serious gram-negative infections are healthcare-associated, and the most common pathogens are *Enterobacteriaceae*, *P. aeruginosa,* and *Acinetobacter.* /CDC, 2013/.

Military Medical Academy /MMA/ in Sofia, Bulgaria, is a community hospital with 800 beds. The hospital is a one of the national centres for treatment of trauma, respiratory disease, liver transplant patients. Antibiotic stewardship at the MMA includes all groups of antibiotics, together with carbapenems, quionolones, third and fourth generation cephalosporins.

Multidrug-Resistant *E. coli* and *Klebsiella* pneumoniae

Multidrug-resistant /MDR/, extended-spectrum beta-lactamases (*ESBLs)* producing *Klebsiella* species and *Escherichia coli* have been isolated in hospitals throughout the world. *ESBLs* positive strains are associated with increased mortality, because of the failure to treat infections caused by *ESBLs* positive organisms, due to the limited therapeutic choices (Kim *et al.,* 2002; Paterson *et al*., 2005). Of all "EARSS-specific" pathogens, *E. coli* demonstrated the most worrying trends. *E. coli* isolates with multiple resistance to third generation cephalosporins, fluoroquinolones, and aminoglycosides were registered in Bulgaria in great proportions (EARSS, 2002). The proportion of ESBL-producing *E. coli* strains in the units of the MMA in 2014 was 22.4% and for *K. pneumoniae* – 47%. Most of the *E. coli* strains, producing ESBL, originated from the so-called "risky units" – ICU (Intensive care unit) 29.9%, Anestesiology and Resuscitation clinic /ARC/ – 17.7% and Hepato-Pancreatic surgery /HPS/ – 16.2%. /MMA, 2015./ In a study of bloodstream infections, the proportion of E.coli producing ESBLs increased from 40% in 2002 to 61% in 2009, and the proportion of carbapenem-resistant *K. pneumoniae* increased from 2.4% to 52% (Datta *et al.,* 2012). The resistance patterns of the *E. coli* and *K. pneumoniae* strains isolated in 2014 are presented in Figures 1 and 2.

ESBLs are clearly a matter of global concern. In Europe*, CTX-M ESBLs*, which began to disseminate clinically later than the classical TEM and SHV variants, are now spreading rapidly and are increasingly dominant. While they were found among hospitalized patients and in species more common in the ICU (*K. pneumoniae, E. cloacae*), *ESBLs* are now commonly found in *E. coli* strains even in patients with community-acquired infections (Corgnaglia *et al*., 2008).

The results of a comprehensive study conducted in Bulgaria over a 8-year period /1996-2003/, showed that *CTX-M-3, CTX-M-15* and *SHV-12*, are the most widespread *ESBLs* in *Enterobacteriaceae* strains, especialy in the *E. coli* and *K. pneumoniae* strains investigated (Markovska *et al.,* 2008). Detection of *E. coli* strains, carriers of *CTX-M-15* in the MMA later in 2012 (Poirel *et al.*, 2014) and in 2014 (Pfeifer *et al*., 2015/, suggested persistence of such problematic hospital isolates over a long period of time in the hospital, requiring constant monitoring and control on ESBL-producing *Enterobacteriaceae* strains, because of the importance of the problem, related to the limited options for treatment of such infections (Mshana *et al*., 2011).

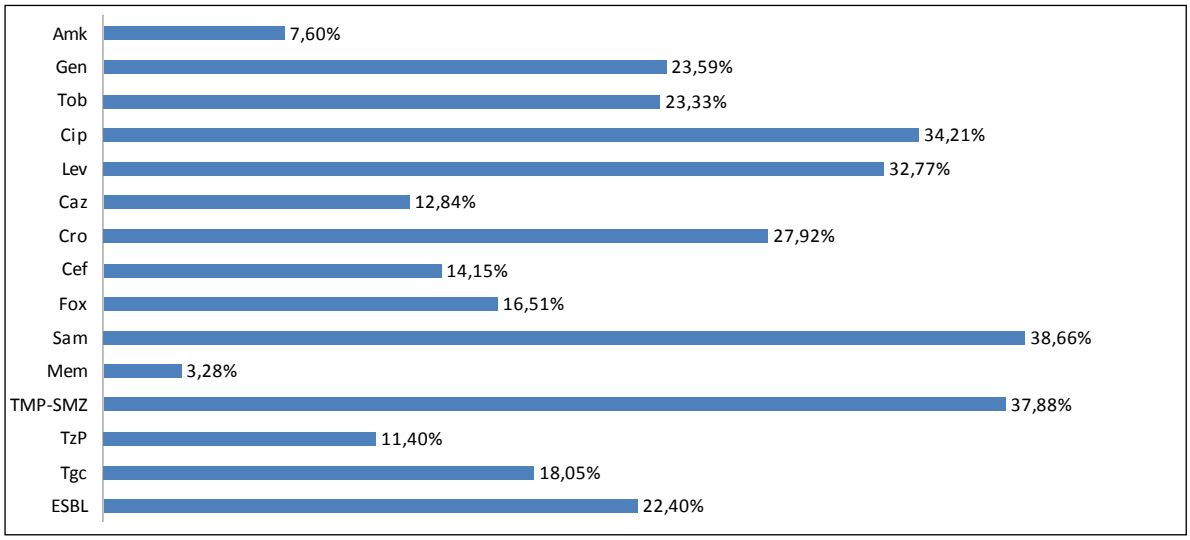

Fig. 1. Resistance of *E. coli* strains to antimicrobials for 2014 amk – amikacin, gen – gentamicin, tob – tobramycin, cip – ciprofloxacin, lev – levofloxacin, caz – ceftazidim, cro – ceftriaxon, cef – cefepim, fox – cefoxitin, sam – ampicillin/ sulbactam, mem – meropenem, tmp-smz – trimetoprim/sulfametoxazol, tzp – piperacillin/tazobactam, tgc - tigecyclin, n-763

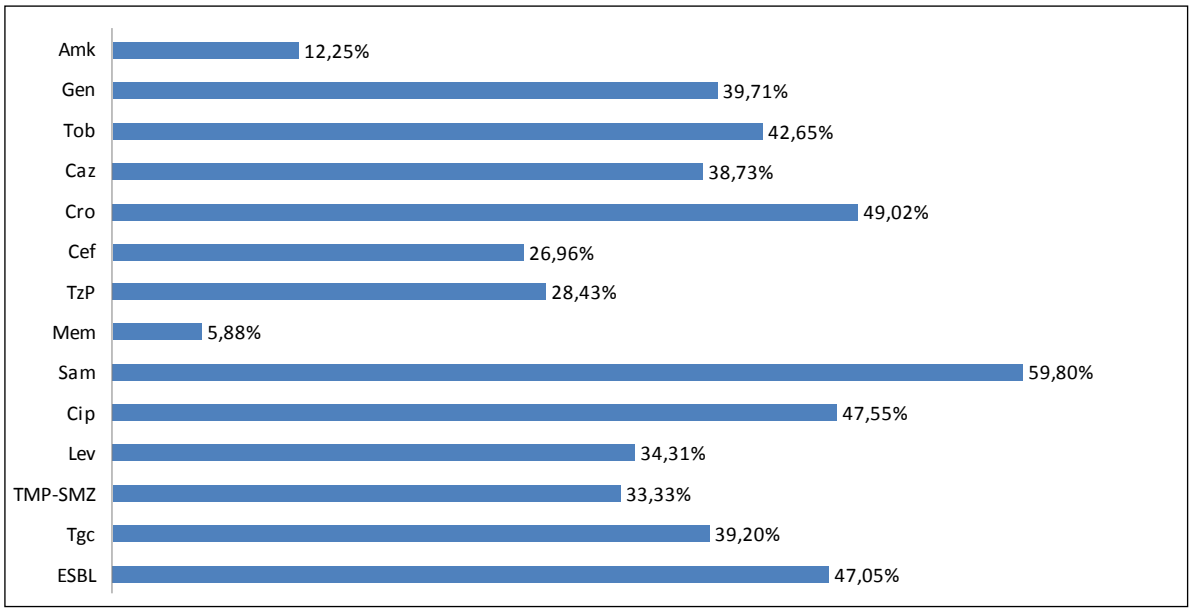

Fig. 2. Resistance of *K. pneumoniae* strains to antimicrobials for 2014 amk – amikacin, gen – gentamicin, tob – tobramycin, cip – ciprofloxacin, lev – levofloxacin, caz – ceftazidim, cro – ceftriaxon, cef – cefepim, fox – cefoxitin, sam – ampicillin/ sulbactam, mem – meropenem, tmp-smz – trimetoprim/sulfametoxazol, tzp – piperacillin/tazobactam, tgc - tigecyclin, n-204

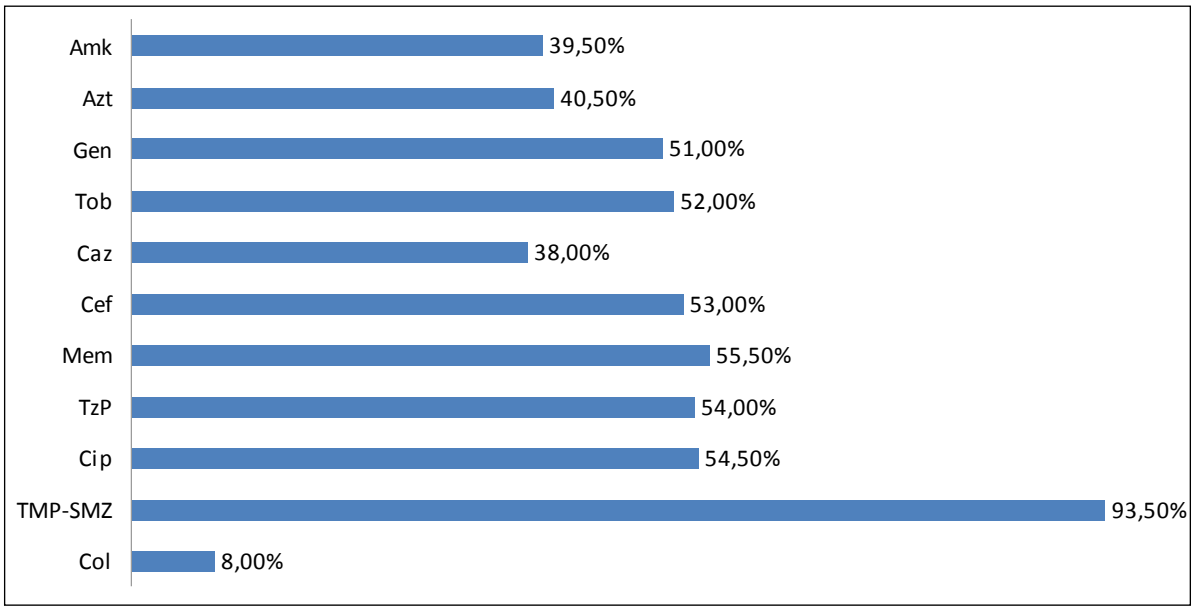

Fig. 3. Resistance of *A. baumannii* strains to antimicrobials for 2014 amk – amikacin, azt – azreonam, gen – gentamicin, tob – tobramycin, caz – ceftazidim, cef – cefepim, cip – ciprofloxacin, sam – ampicillin/sulbactam, mem – meropenem, tzp – piperacillin/tazobactam, tmp-smz – trimetoprim/sulfametoxazol, tgc – tigecyclin, col – colistin, n-200

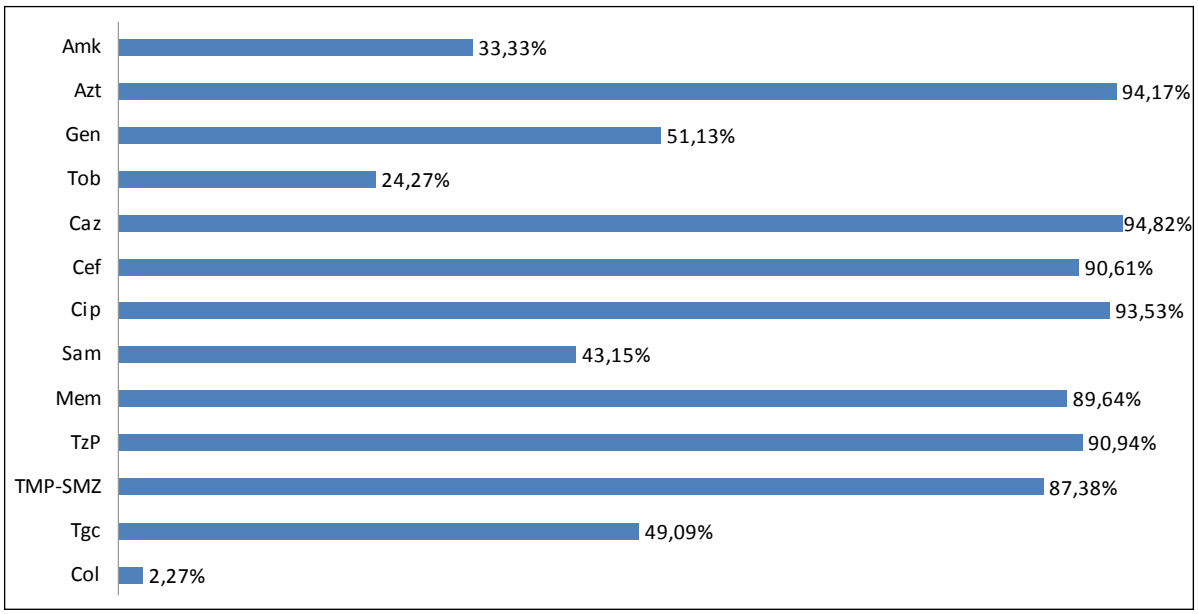

Fig. 4. Resistance *of P. aeruginosa* strains to antimicrobials for 2014 amk – amikacin, azt – azreonam, gen – gentamicin, tob – tobramycin, caz – ceftazidim, cef – cefepim, mem – meropenem, tzp – piperacillin/tazobactam, cip – ciprofloxacin, tmp-smz – trimetoprim/sulfametoxazol, col – colistin, n-309

Carbapenem resistance among common *Enterobacteriaceae* has increased sharply over the past decade. The proportion of *Enterobacteriaceae* resistant to carbapenems increased from 0.0% to 1.4% in 2010, with most of the increase recorded in *Klebsiella spp*. (CDC, 2013).

In comparison with 2008, when the resistance to meropenem registered for *E. coli* was 0.0% and for *K. pneumoniae* - 1.4%, the data for 2014 showed an increase of 3.3% for *E. coli* and 5.9% for *K. pneumoniae*, respectively. In Europe, there are distinct epidemiological situations, corresponding mainly to the diffusion of *OXA-48* producers in France, Belgium, the Netherlands and Turkey, and *KPC* – producing isolates in Italy and Greece (Poirel *et al.,* 2014). In contrast, in 2012 the first NDM-1 carbapenemase-producing *E. coli* strains were described in Bulgaria, together with the extended-spectrum-lactamase *CTX-M-15* and the *16S rRNA* methylase *RmtB*, conferring high-level resistance to all aminoglycosides (Savov *et al.,* 2012; Poirel *et al.*, 2014). All the isolates were clonally related and belonged to the same sequence type, ST101. In addition to being the first to identify *NDM*-producing isolates in Bulgaria, this was the very first study reporting an outbreak of *NDM-1*-producing *E. coli* in the world (Poirel *et al.,* 2014). The recent information on *NDM-1* producers /mostly *K. pneumoniae* isolates/ has often been related to imported cases, with a link to the Indian subcontinent. Therefore, it is very important to note that Bulgarian *E. coli* strains, positive for NDM-1 production, together with those, isolated in Croatia (Mazzariol *et al*., 2012), Kosovo (Struelens *et al*., 2010), Serbia (Jovci *et al*., 2011), Bosnia and Herzegovina (Struelens *et al.,* 2010) and Montenegro (Struelens *et al*., 2010), suggest that the Balkan region is probably another area of endemicity in addition to the Indian subcontinent.

Aminoglycoside resistance has become an expected companion to the NDM-1 beta-lactamase in almost every isolate, but this specific association between the *blaNDM*-1 and *rmtB* genes has been very rarely reported, and mainly in *E. coli*, with a single isolate from Australia (Poirel *et al.,* 2010) and a single isolate from Belgium (Pakistan origin) (Bogaerts *et al.,* 2011). *VIM* producing organisms also exhibit high resistance to aminoglycosides, due to aminoglycoside-modifying enzymes such as acetyltransferases encoded by genes such as *aacA4* found in *P. mirabilis* strains, isolated at MMA in 2014 (Pfeifer *et al.,* 2015). Unfortunately, in many cases most carbapenemases were found with a number of other resistance factors that encode resistance to almost all classes of antibiotics, used to treat infections caused by Gram-negative bacteria and most affected were the aminoglycosides and fluoroquinolones, antibacterials that have been used widely as therapeutic options for variety of Gram-negative infections (Bush, 2013).

Multidrug (pandrug)-resistant *Acinetobacter baumannii* infections

A. baumannii has emerged as one of the most troublesome pathogens for health care institutions on a global scale (Davis *et al*., 2005; Peleg *et al*., 2008; Savov *et al*., 2015). Its clinical significance, especially over the last 15 years, has been propelled by its remarkable ability to acquire resistance determinants, making it one of the organisms threatening the current antibiotic era (Davis *et al*., 2005). The rapid global emergence of *A. baumannii* strains resistant to all β-lactams, including carbapenems, quinolones and other antimicrobials, illustrates the potential of this organism to respond swiftly to changes in selective environmental pressure (Peleg *et al.,* 2008). After performing whole-genome sequencing of a clinical epidemic *A. baumannii* strain found in France /AYE/, an 86-kb resistance island, one of the largest to be described thus far, was identified /*AbaR1/.* Overall, 52 resistance genes were identified, and surprisingly, 45 (86.5%) were localized in the *AbaR1* resistance island (Fournier *et al*., 2006).

Resistance to carbapenems at the MMA in Sofia (89% to meropenem) (Fig. 3) is associated with the production of *Oxa 23* and *Oxa 58* carbapenemases, but not to metallo-beta-lactamases (Stoeva *et al.,* 2009; Savov *et al.,* 2010). The resistance to quinolones was assessed at the DNA level for mutation detection in quinolone-resistance-determining regions /QRDRs/ and the subsequent aminoacid substitution in the *GyrA* and/or the *ParC* enzymes. A strong correlation was found between quinolone resistance and mutations *in gyrA codon 83* and/or in the *parC* gene /*codons 80 or 84*/ (Deccache *et al.*, 2011).

Recently, we have isolated a *A. radioresistens* strain, which can be considered as a cause of opportunistic infection in immunocompromised patients. Additionally, we found that the strain harboured the carbapenemase gene *blaOXA-23* without insertion sequences upstream of this gene but with the sensitivity to imipenem and meropenem (Savov *et al*., 2015). It is not uncommon since *A. radioresistens* is known as a silent source of carbapenem resistance

owing to chromosomally encoded *blaOXA-23*-like genes lacking insertion sequences as described previously (Poirel *et al*., 2008; Boo *et al*., 2009).

Multidrug-resistant *Pseudomonas aeruginosa* infections

P. aeruginosa is a non-fermenting Gram-negative microorganism, which is a major dreaded cause of infection among hospitalized patients, usually with localized or systemic impairment of immune defence. It is a common cause of hospital-acquired infections like pneumonia, urinary-tract infections, wound infections, respiratory tract infections, bloodstream infections, especially in the units with high risk for infection development (Jalal *et al*., 2000; ECDC, 2010). According to our data for 2014, *P. aeruginosa* occupies the 6th place with 6.4% among the first 10 most frequently isolated microorganisms in the MMA and with 15% isolation in ICU, as well (MMA, 2015). *P. aeruginosa* is resistant to the majority of antimicrobial compounds due to its selective ability to exclude various molecules from penetrating its outer membrane and involvement of an active efflux mechanism. It is a problem, because in many cases the *P. aeruginosa* strains isolated were multiresistant - with resistance to: piperacillin/ tazobactam of 54%, cefepime - 53%, and ceftazidime - 38%. The level of the resistance to meropenem is 55% (Fig. 4).

The spread of similar multiresistant strains is very important for big hospital complexes also according to Edalucci et al*.,* 2008. This multiresistance is usually connected with production of metallo-beta-lactamase (MBL) *VIM-2* and also these widespread clones, responsible for human infections, belong to O11 and O12 serotypes (Edalucci *et al.*, 2008). Another problem which is very important for the treatment of infections caused by these microorganisms is the resistance development to quinolones in the last years. Evolution at the molecular level involves the gradual accumulation of mutations /and other changes/ in DNA sequences. The major mechanism of resistance of this bacterium to fluoroquinolones is the modification in *gyrA* gene supplemented by possible changes in *parC* and *mexR* regions. In this sense, we reported the detection of point mutations in *codon 83* of *gyr A* gene, *codons 87* and *136* of the *parC* and *codons 126* and 44 of the *mex*R regulatory gene. Mutations in *gyr A* gene were found in all *P.aeruginosa* strains, resistant to ciprofloxacin (Savov *et al*., 2014).

Perspectives, cooperation against resistant bacteria

Antibiotic resistance, especially in Gram-negative bacteria, is a global problem with many examples of the rapid spread of new resistance between continents. Laxminarayan and colleagues warn that "we are at the dawn of the postantibiotic era", with "almost all disease-causing bacteria resistant to the antibiotics commonly used to treat them" (Laxminarayan *et al.,* 2013). Recent routine surveillance of antibiotic resistance in commensal *E. coli* from food animals in China has documented a major increase of colistin resistance due to a highly mobile, transferrable, plasmid-mediated colistin-resistance gene designated *mcr-1* with the possibility to pass to *K. pneumoniae* and even to *P. aeruginosa* strains. (Liu *et al*., 2016). Later similar *E. coli* strains were isolated from patients, which means that we may soon face a situation without useful antibiotics to treat infections caused by MDR Gram-negative bacteria (Coetzee *et al*., 2016; Prim *et al*., 2016). This multidrug-resistance affects the practice in many different medical disciplines and resolution needs coordination of efforts of the different medical specialists and pharmacists. It is very important and in this connection at the EU-US Summit on November 3, 2009 in Washington, President B. Obama, Jose Manuel Barroso, Fredric Reinfeldt, and Javier Solana agreed to establish a transatlantic task force on urgent antimicrobial resistance issues /EU-US Summit agrees to form transatlantic task force on antimicrobial resistance /www.reactgroup.org/. The task force has to focus on appropriate therapeutic use of antimicrobial drugs in the medical and veterinary communities, prevention of both healthcare and community-associated drug resistant infections, and strategies for improving the pipeline of new antimicrobial drugs, which could be better addressed by intensified cooperation between the US and Europe. Following this, the IDSA Antibiotic Availability Task Force announced the necessity to achieve the development of ten new antibiotics within the next ten years (the 10 × '20 initiative), meaning that the aim is to develop 10 novel drugs for Gram-negative bacteria by the year 2020 /www.reactgroup.org, http://www.medscape.com/. Further, the key elements to be used in this aspect include improvement of infection prevention and control practices in human and animal health, improvement of professional education, training and public engagement, better access to and use of surveillance data, better identification and prioritization of research into antimicrobial resistance and

strengthened international collaboration (Howard *et al*., 2013).

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VanA and *MecA* Genes in *Staphylococcus aureus* Isolates in North-Eastern Iran

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Abstract

The increasing number of methicillin-resistant *Staphylococcus aureus* (MRSA) infections strains is a global health threat. Vancomycin is one of the very limited options in treating such infections. The emergence of vancomycin-resistant *S. aureus* (VRSA) is therefore a great concern in clinical settings. During recent years, the incidence of vancomycin-intermediate *S. aureus* (VISA) and vancomycin-resistant *S. aureus* has increased in various parts of the world, which have been identified based on criteria defined by the Clinical and Laboratory Standards Institute (CLSI). We have recently shown a high resistance rate to methicillin in *S. aureus* isolates from two main university hospitals in northeastern Iran. Here we expanded the study to reveal the frequency of *vanA* and *mecA* genes in the isolated MRSA strains. We selected 45 MRSA isolates, which were shown phenotypically methicilin- resistant to further genotypic investigation of the *mecA* and *vanA* genes. DNA was extracted from bacterial suspension and *mecA* and *vanA* genes were identified using PCR technique. The majority of MRSA isolates, 42 out of 45 (93%), were positive for the *mecA* gene. None of the MRSA isolates were positive for the *vanA* gene.

The *mecA* gene is frequently circulating among phenotypically identified MRSA isolates, which confirms the phenotypically resistant strains and explains the resistance mechanism. The high frequency of circulating *mecA* gene highlights the need for policies to overcome the MRSA problem in clinical settings. Though none of the isolates showed vancomycin-resistance based on phenotypic tests, we also evaluated the isolates for possible *vanA* gene positivity and none of the isolates were shown to be positive for the *vanA* gene.

Key words: *Staphylococcus aureus*, methicillin, vancomycin, resistance gene

Резюме

Нарастващият брой инфекции с метицилин-резистентни *Staphylococcus aureus* (MRSA) представлява глобален здравен риск. Една от малкото възможности за третиране на тези инфекции е с ванкомицин. Поради това появата на ванкомицин-резистентни *S. aureus* (VRSA) е сериозен проблем в клиничната практика. През последните години в различни части на света се увеличи честотата на щамове *S. aureus* с междинна чувствителност към ванкомицин (VISA) и на ванкомицин-резистентни *S. aureus*, което е доказано на базата на критериите, дефинирани от Института по клинични и лабораторни стандарти (CLSI). Наскоро ние показахме високо ниво на резистентността при *S. aureus*, изолирани в двете основни университетски болници в североизточен Иран. В настоящата работа изследването е разширено с цел доказване на честотата на гените *vanA* и *mecA* при изолираните щамове MRSA. Избрахме 45 изолирани щама MRSA, които фенотипно показват резистентност към метицилин, за по-нататъшно изследване на гените *mecA* и *vanA*. От бактериални суспензии екстрахирахме ДНК и идентифицирахме гените *mecA* и *vanA* чрез метода PCR. Повечето изолати MRSA, 42 от общо 45 (93%) бяха позитивни за гена *mecA*.

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Нито един от изолираните MRSA не беше позитивен за гена *vanA*.

Генът *mecA* често циркулира между изолатите, идентифицирани фенотипно като MRSA, което потвърждава тяхната фенотипна резистентност и обяснява механизма на резистентността. Високата честота на гена *mecA* подчертава необходимостта за политики, насочени към преодоляване на проблема с MRSA в клинични обстоятелства. Въпреки че при фенотипните тестове нито един от изолатите не показа резистентност към ванкомицин, ние направихме проверка и за наличието на *vanA* гена, но нито един от изолатите не беше позитивен за този ген.

Introduction

Staphylococcus aureus is the most important human pathogen among the genus of *Staphylococcus*. *S. aureus* pathogenicity can cause a wide range of illnesses, from skin infections to severe conditions, such as sepsis, endocarditis, osteomyelitis, pneumonia, etc. (Harris *et al.,* 2002; Plata *et al*., 2009). This bacterium is the main cause of hospital and community-acquired infections (Plata *et al*., 2009; Al-Obeid *et al.,* 2010). Owing to high morbidity and increasing resistance against a wide range of antibacterial drugs, this bacterium has become one of the major public health concerns in all clinical settings worldwide.

Methicillin was introduced in the late 1950s as a good choice to treat life-threatening *S. aureus* infections, however, the widespread usage of this antibiotic caused numerous methicillin-resistant *S.aureus* reports (Tong *et al.,* 2012). The increasing number of MRSA strains in hospitals and communities, and more importantly, the emergence of multidrug-resistant (MDR) MRSA, led to the use of vancomycin in the treatment of MRSA infections (Tiwari *et al*., 2009; David and Daum, 2010).

However, reports of MRSA isolates with reduced susceptibility to vancomycin raised the first alarms about vancomycin-resistant *S. aureus (VRSA)* (Périchon and Courvalin, 2009; Jacob and DiazGranados, 2013). One strain with reduced susceptibility to vancomycin was first reported from Japan in 1996 (Hiramatsu, 2001; David and Daum, 2010). Based on interpretive criteria defined by the Clinical and Laboratory Standards Institute (CLSI), the vancomycin minimum inhibitory concentration (MIC) result reported for the new isolate was in the intermediate range (8μg/mL) (Saderi *et al*., 2005). Shortly after, in 2002, the first clinical vancomycin-resistant *S. aureus* (MIC ≥32μg/mL) strain was isolated in Michigan, USA (Dezfulian *et al*., 2012; Hiramatsu *et al*., 2014). Afterwards, several reports of vancomycin-intermediate *S. aureus* (VISA) and VRSA from different parts of the world provoked a growing concern about the success of vancomycin therapy in critical staphylococcal infections (Howden *et al*., 2010; Azimian *et al.,* 2012). Two main resistance mechanisms have been proposed: i) thickened and poorly cross-linked cell wall for vancomycin intermediate-resistant *S. aureus*, ii) activity of *van A* operon. This operon acquired from *Enterococcus* spp results in high-level resistance and defines vancomycin-resistant *S. aureus* (Hiramatsu, 2001; Périchon and Courvalin, 2009; 2012; Tarai *et al.,* 2013).

The higher incidence of MRSA in different parts of the world may lead to a higher frequency of prescribing vancomycin, which in turn may cause emergence of VRSA. We have recently shown a high resistance rate to methicillin in *S. aureus* isolated in two main university hospitals in northeastern Iran (Rahimipour *et al*., 2015). Here we expand the study to reveal the frequency of *vanA* and *mecA* genes in these isolates.

Material and Methods

Bacterial isolates

The isolates were obtained from clinical samples as described before (Rahimipour *et al*., 2015). We selected 45 MRSA isolates which were previously shown phenotypically methicilin-resistant based on E test and MIC determination. The strains were subjected to further genotypic investigation of the *mecA* and *vanA* genes. Though none of the isolates showed vancomycin resistance in phenotypic study, we also evaluated the isolates for possible *vanA* gene positivity.

DNA extraction

DNA was extracted from bacterial suspension using a DNA extraction kit (Genomic DNA isolation kit VI, DENAzist Asia/Mashhad, Iran) according to manufacturer's instructions.

Primers

We used two primer pairs to detect the *mecA* and *vanA* genes as described before (Azimian *et al.,* 2012). The forward (F) and reverse (R) primers were as follows: F1: 5'AGAAGATGGTATGTGGAAGTTAG3'and R1: 5'ATGTATGTGCGATTGTATTGC3' and F2: 5'GGCAAGTCAGGTGAAGATG3' and R2: 5'ATCAAGCGGTCAATCAGTTC3' for *mecA* and *vanA* genes, respectively.

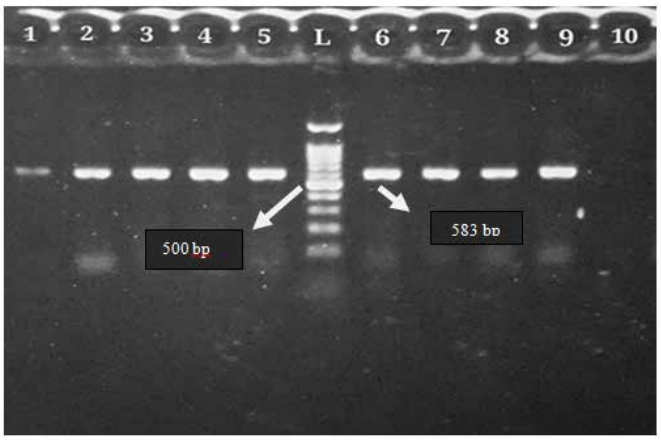

Fig. 1. *MecA* gene pattern of agarose gel (1.5%) electrophoresis. Lane 1-8 clinical strains, Lane 9 positive control, Lane 10 negative control, Lane L DNA marker.

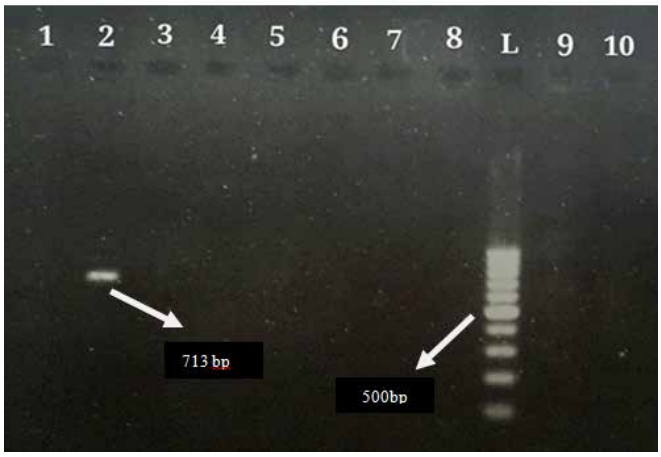

Fig. 2. *Van A* gene pattern of agarose gel (1.5%) electrophoresis. Lane 1 negative control, Lane 2 positive control, Lane 3-10 clinical strains, Lane L DNA marker

PCR reaction conditions

The PCR reaction was optimized by applying concentration and temperature gradients. Finally a 25 µl reaction consisted of: 5 µl template DNA, 0.2 µl DNA polymerase (Takapouzist, Tehran), 2 µl primer (100 Pmol), 0.5 µl dNTPs (200µM), 2 µl MgCl2 (1.5mM), 2.5 µl PCR buffer (10X) and 12.8 µl DDW. The final PCR program was determined using gradient PCR optimization. The final program was set at the following conditions: 5 min at 94°C for initial denaturation, followed by 35 cycles consisting of denaturation at 94°C for 1 min for *mecA* and 1 min for *van A*, annealing at 45°C for 1 min for *mecA* and at 46°C for 1 min for *van A*, and extension at 72°C for 1 min for *mecA* and 90 s min for *vanA*, with a final extension step at 72°C for 5 min. Next, the PCR products were subjected to 1% agar gel electrophoresis. The gels were stained and visualized with an UviDoc system. The PCR products were finally sent for DNA sequencing with the above mentioned primers.

Results

A 583 bp fragment corresponding to *mecA* and a 713bp for *vanA* were observed on DNA gel electrophoresis of PCR products (Fig. 1, Fig. 2, respectively). Among all MRSA isolates, 42 out of 45 (93%) were positive for the *mecA* gene. None of the MRSA isolates were positive for the *vanA* gene.

The sequenced PCR products were aligned with the sequences of the *mecA* gene using Nucleotide BLAST (Basic Local Alignment Search Tool) available in NCBI database and a homology of >96.% was observed.

Discussion

MRSA infections are considered as a main concern in hospital settings all around the world. Among these infections, MRSA bacteremia has a higher mortality rate (almost double) than methicillin-susceptible *S. aureus* (MSSA) bacteremia (Moise-Broder et al., 2004). It has been also reported that MRSA isolates are mostly multidrug-resistance (Sharif *et al*., 2013; Tiwari *et al.,* 2009). Similarly, the majority of our MRSA strains were multidrug-resistant to other antibiotics including penicillin, gentamicin, clindamycin and erythromycin (Rahimipour *et al.*, 2015). In this regard, one could imagine that VRSA strains tend to have simultaneous resistance against a large number of other antibiotics, resulting in a narrow treatment option and higher morbidity and mortality (Thati *et al.,* 2011).

In our geographic region, we have recently reported that 45 out of a total of 122 strains (36.88%) were MRSA strains (Rahimipour *et al.,* 2015).

The genetic mechanisms of methicillin and vancomycin resistance in MRSA and VRSA are related to *mecA* and *vanA* genes, respectively. According to previous studies, the *vanA* gene can be easily transferred from *Enterococci* to *S. aureus* (Périchon and Courvalin, 2009). In this study, 42 isolates contained the *mecA* gene, but all strains were negative for the *vanA* gene. The high frequency of circulating *mecA* gene among *S. aureus* strains alarms for possible emergence of VRSA in upcoming years. This underscores the need for careful strategies for

managing such possible health system problem. During recent decades, the increasing prevalence of methicillin-resistant *S. aureus* in many parts has resulted in a dramatically increased use of vancomycin (Tiwari *et al*., 2009; Rao and Prabhakar, 2011). Such prescriptions should be used with utter care. For example, suitable vancomycin dosing to ensure complete destruction of bacteria has been emphasized. Additionally, the use of combination therapy against MRSA should not be ignored (Shahriar *et al*., 2012).

To summarize, based on the present study and previous published studies *mecA* gene is widely detected in *S. aureus* strains, which may lead to increased treatment with vancomycin and ultimately may result in emergence of VRSA strains. Therefore, an urgent response is essential to restrain further spread and emergence of resistant strains. With this regard, a precise protocol on proper antibiotic use is needed in all clinical settings and agents such as vancomycin should be used in particular conditions, when it is absolutely necessary.

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In Vitro Anti-Rhinovirus Activity of Some Picornavirus Replication Inhibitors

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Abstract

The effect of several antiviral substances with different mode of action on the replication of human rhinovirus 14 (HRV-14) is the topic of the present study. Monolayer cultures of human cervical carcinoma (HeLa Ohio-I) cells in 96-well tissue culture plates were used. The antiviral effect against three different viral inoculation doses was tested by the neutral red uptake procedure in a CPE-inhibition setup. The following compounds were tested: ribavirin, arildone, disoxaril, S7, PTU-23, HBB and oxoglaucine (a newly characterized in this laboratory compound efficient against enteroviruses). Two of the compounds, HBB and oxoglaucine, showed the highest activity with a selectivity ratio (CC_{50}/IC_{50}) above 100 for the lowest viral inoculation dose of 100 $CCID_{50}$. Ribavirin and disoxaril occupied intermediate position according to their antiviral effect, while the effect of arildone, PTU-23 and S-7 was not significant.

Key words: Rhinovirus H14; Ribavirin; Oxoglaucine; HBB; Disoxaril

Резюме

Предмет на настоящето проучване е ефектът на серия антивирусни вещества с различен начин на действие върху репликацията на човешки риновирус 14 (HRV-14). Използвани бяха монослойни култури на клетки от човешки цервикален карцином (HeLa Ohio-I) в 96-ямкови тъканно-културални плаки. Антивирусният ефект на три различни вирусни дози бе изпитан по метода на инхибиране на цитопатичния ефект чрез процедурата на поглащане на неутрално червено. Тестирани бяха следните съединения: рибавирин, арилдон, дизоксарил, S7, PTU-23, HBB и оксоглауцин (ново съединение, охарактеризирано в нашата лаборатория като ефикасно срещу ентеровируси). Две от съединенията, HBB и оксоглауцин, показаха най-висока активност с индекс на избирателност (CC_{50}/IC_{50}) над 100 при най-ниската вирусна инокулационна доза 100 $CCID_{50}$. Рибавирин и дизоксарил заеха междинна позиция според антивирусния им ефект, докато ефектът на арилдон, PTU-23 и S7 бе статистически недостоверен.

Introduction

Picornaviruses are the most common cause of viral illness worldwide (Rotbart and Hayden, 2000). This family of small single-stranded RNA viruses currently comprises nine genera, including *Enterovirus* and *Rhinovirus*. In 2004, a proposal was submitted to the International Committee on Taxonomy of Viruses to merge the genera *Rhinovirus* and *Enterovirus* into a single genus, *Enterovirus*, with the species in it remaining intact and under the banner of a new virus order, *Picornavirales* (Le Gall *et al*., 2008). Human rhinoviruses (HRVs) comprise over 150 different virus serotypes. HRVs are the predominant cause of viral upper respiratory tract infections and particularly of common cold (Arruda *et al*., 1997; Couch, 2001; Turner, 2001)

These infections are often mild and self-limiting; nevertheless, they have a significant socioeconomic impact (Patick, 2006). Increasing evidence also describes the link between HRV infection and more serious medical complications like acute otitis media, sinusitis, pneumonia and bronchiolitis in infants and young children. Rhinoviral infection commonly causes exacerbations of the pre-existing airways disease in those with asthma, chronic obstructive pulmonary disease or cystic fibrosis, (Pitkaranta and Hayden, 1998; Bardin, 2004; Gern, 2004; Hayden, 2004; Tan, 2005; Khetsuriani *et al.*,

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2007). Among elderly people, infants and highly immunocompromised hosts, HRV infections are associated with morbidity of the lower respiratory tract and rarely mortality (Pitkaranta and Hayden, 1998; Hayden, 2004) .

To date no effective antirhinoviral chemotherapy has been approved for clinical use and the treatment of these infections is limited to symptomatic therapy only (Savolainen *et al*., 2003; Turner, 2005; Patick, 2006).

In this study we describe the results of cell culture studies on the anti-rhinovirus activity of seven picornavirus replication inhibitors: HBB (2-α-hydroxybenzyl-benzimidazole), oxoglaucine, ribavirin, disoxaril, arildone, PTU-23 (N-phenyl-N'-3-hydroxyphenylthiourea) and S-7 (ethyl-2-methylthio-4-methyl-5-pyrimidine carboxylate).

Materials and Methods

Cells

Human cervical epithelioid carcinoma (HeLa Ohio-I) cells were a kind gift of Dr. D. Barnard (Utah State University, Logan, USA). The cells were grown in minimal essential medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco), sodium hydrogen carbonate at a final concentration of 25 mM, 10 mM HEPES buffer (AppliChem GmbH, Darmstadt, Germany), 50 IU of penicillin, 50 μg/ml of streptomycin and 50 μg/ml of gentamicin in a 5% CO_2 incubator HERA cell 150 (Heraeus, Hanau, Germany).

Virus

Human rhinovirus type 14 (strain 1059) (HRV-14) was used for the experiments described. The virus was purchased from the American Type Culture Collection (Manassas, VA, USA). HRV-14 stocks were prepared in HeLa Ohio-I cells in a maintenance medium. The maintenance medium was like the above described growth medium except for the serum which was reduced to 2 %. Stock virus titer was $10^{6.5}$ $CCID_{50}$/ml.

Compounds

Seven compounds with different mode of action were tested. These were HBB (2-α-hydroxybenzyl-benzimidazole), (a gift from Dr. T. Dmitrieva, Moscow State University, Moscow, Russia); oxoglaucine (Dr. S. Philipov, Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria); ribavirin (1-(β-D-ribofuranosyl)-1H-1,2,4-triazole-3-carboxamide), (a gift from Prof. R. W. Sidwell, Utah State University, Logan, USA); disoxaril (5-[7-[4(4,5-dihydro-2-oxazolyl)phenoxy] heptyl]-3-methylisoxazole; WIN 51711), (Sanofi Winthrop, Inc., PA); PTU-23 (N-phenyl-N'-3-hydroxyphenylthiourea), originally synthesized by Prof. G. Vassilev (Institute of Plant Physiology, Bulgarian Academy of Sciences, Sofia); arildone (4-[6-(2-chloro-4-methoxy-phenoxy)-hexyl] -3,5-heptanedione), (Sterling Research Group, Sterling Drug, Inc., USA); and S-7 (ethyl 2-methylthio-4-methyl-5-pyrimidine, carboxylate), (a gift from Dr. P. La Colla, University of Cagliari, Cagliari, Italy). All drugs, with the exception of ribavirin and PTU-23, were prepared as stock solutions in dimethyl sulfoxide (DMSO) and then diluted in the maintenance medium to the required concentrations. Ribavirin was dissolved directly into the medium and PTU-23 was initially dissolved in ethanol and then in the test medium. In the experiments 0.5 log_{10} dilutions of the compounds were used.

Virus assay

The virus titer was quantified in a 50% cell culture infectious dose ($CCID_{50}$) assay following the endpoint dilution design (Reed and Muench, 1938). HeLa Ohio–I cells were seeded into 96-well tissue culture microplates at 4 x 10^4 cells/well in Minimal Essential Medium (MEM) followed by overnight incubation at 37°C. The growth medium was then removed and serial 10-fold dilutions of virus were added (100 μl/well; eight wells per dilution). After 2h of adsorption at 33°C, excess virus was removed and 0.1 ml of maintenance medium was added to each well. The virus titer was presented as log_{10} of 50% cell culture infectious dose ($CCID_{50}$/ml) by visual recording of the virus cytopathic effect (CPE) following 72h of incubation at 33°C.

Antiviral tests

The end-point dilution method in the multi-cycle cytopathic effect (CPE) inhibition set up was used to assess the antiviral effect of the compounds. Confluent cell monolayers of HeLa Ohio-I in 96-well tissue culture microplates were infected with three viral inoculation doses 100, 1000 and 10 000 $CCID_{50}$ per well or mock infected with maintenance medium only. After 2h of adsorption at 33°C, excess virus was removed and medium containing 0.5 log_{10} dilutions of the test compounds or medium only for the toxicity control was added. Each drug concentration was assayed in quadruplicate. After 3 days of incubation at 33°C, the medium was removed, cells were washed with phosphate-buffered saline (PBS) and then stained with neutral red. The plates were incubated for 3 h at 33°C in the dark

to allow the cells to absorb the dye. After rinsing and drying steps, ethanol/acetic acid solution was added to each well and plates were shaken on a microtiter plate shaker for 10 min until neutral red was extracted from the cells and formed a homogeneous solution. Absorbance at 540 nm was read with a microplate reader (Organon Teknika Reader, Anthos Labtec Instruments GmbH, Salzburg, Austria).

Cytotoxicity assay

Cytotoxicity tests were done in the same plate simultaneously with the antiviral tests. After formation of the cell monolayer, the growth medium was discarded and 0.1 ml containing 0.5 $\log_{10}$ dilutions of the test compounds diluted in maintenance medium was added. The results were read after 72 hour by neutral red uptake procedure.

Statistical analysis

Absorbance values obtained from the antiviral and cytotoxicity tests were expressed as percentage of untreated or uninfected controls and the 50% inhibitory concentration (IC_{50}) and 50% cytotoxic concentrations (CC_{50}) were calculated by regression analysis. Selectivity index (SI) was calculated using the formula $SI = CC_{50}/IC_{50}$.

Trials were carried out in quadruplicate in three to five independent experiments. Virus titers were calculated according to Reed and Muench (1938). Mean values and standard deviations, as well as IC_{50} and CC_{50} were calculated by regression analysis accomplished with Origin 7.5 computer program.

Results and Discussion

The cytotoxic concentrations 50 (CC_{50}) of all seven compounds tested for HeLa Ohio–I cell line were determined. Fig.1 represents the amount of viable cells as percent of the control depending on the concentration of each compound. As seen, S-7, HBB and ribavirin possess lowest cytotoxicity (CC_{50} values exceeding 4 mM), PTU-23 occupies intermediary position and disoxaril, arildone and oxoglaucine have CC_{50} values below 55 μM.

The activities of all tested compounds against HRV-14 are expressed as 50% inhibitory concentrations (IC_{50}). The results are determined from the dose-response curves obtained by the CPE inhibition test for each compound at three viral inoculation doses. The data are summarized in Table 1.

To estimate the selectivity of the antiviral action of tested compounds, the data for inhibitory concentration and cytotoxicity of each compound and viral inoculation dose are used. The calculated selectivity ratio is displayed in Table 2.

2-(α-hydroxybenzyl)benzimidazole (HBB) is a compound known for decades as a selective picornavirus inhibitor (Tamm and Eggers, 1962; Dmitrieva and Agol, 1974). It is known that this substance, at a concentration nontoxic to cells, inhibits the synthesis of viral RNA (Eggers and Tamm, 1961; Dmitrieva and Agol, 1974). Tamm and Eggers (Tamm and Eggers, 1962) have recognized the considerable variation in the susceptibility of different picornaviruses to the action of HBB. Thus, most enteroviruses are found to be sensitive to inhibition by HBB, whereas many rhinoviruses are found insensitive (Tamm, 1972). More recent studies on the mechanism of action of HBB have revealed that the compound interacts directly or indirectly with the nonstructural protein 2C (Hadaschik *et al.*, 1999). Although many rhinoviruses are found insensitive to it, HRV-14 is one of the few, which is inhibited by 200-220 μM of HBB (Gwaltney, 1968). In our study the effect of the compound against human rhinovirus 14 is confirmed but using a different approach, namely the neutral red uptake assay. The data presented in Table 1 show that concentrations of 44 μM, 128 μM and 179 μM are sufficient to protect 50% of the cells from 100, 1000 and 10 000 $CCID_{50}$, respectively. The inhibition depends on the concentration of the compound as well as on the viral inoculation dose. Compared to the results obtained for the other tested compounds, the effect of HBB is well pronounced and comparable to the data for the effect of oxoglaucine.

Oxoglaucine is an aporphinoid alkaloid isolated from the aerial parts of the plant *Glaucinum flavum* Cranz (Kuzmanov, 1992). It can be obtained synthetically from the main plant alkaloid (Philipov *et al*., 1998). This compound has been found to be active against a panel of 16 enteroviruses with some variations in the sensitiveness of the different enteroviruses (Galabov *et al.*, 1995; Nikolaeva-Glomb *et al*., 2008). The results summarized in Table 1 reveal the marked inhibitory effect of oxoglaucine on the replication of human rhinovirus 14. The compound inhibits all three virus inoculation doses tested. The values of selectivity ratio calculated for oxoglaucine and HBB (data are shown in Table 2) are very similar. The comparison between the antiviral effects of HBB and oxoglaucine allows the statement that oxoglaucine can be situated among the most effective antipicornaviral compounds.

Ribavirin is a potent antiviral agent active against different viruses. In some cases, this inhibition has transferred into clinical applications. Five distinct mechanisms have been proposed to explain

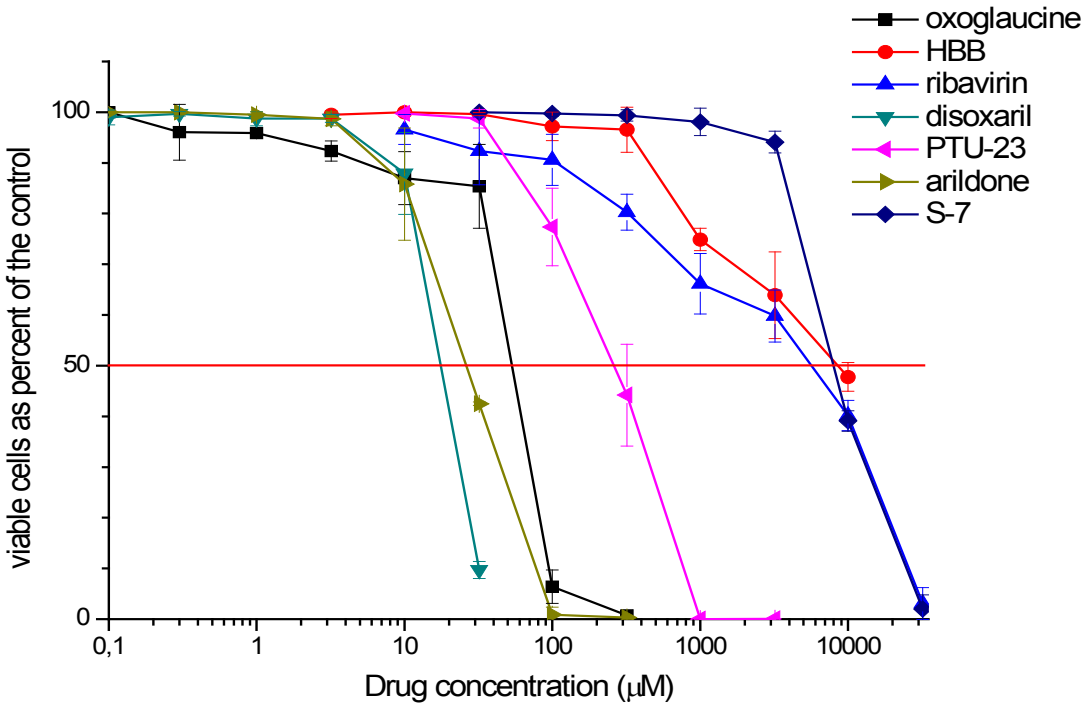

Fig.1. Cytotoxicity of HBB, oxoglaucine, ribavirin, disoxaril, PTU-23, arildone and S-7 determined according to the neutral red uptake procedure

Table 1. Anti-rhinovirus activity of seven antiviral agents

Viral inoculation dose	Antiviral activity (IC_{50} [μM])[a] of:						
	HBB	Oxoglaucine	Ribavirin	Disoxaril	PTU-23	Arildone	S-7
100 $CCID_{50}$	44	0.390	116	1.538	50	9.770	2484
1000 $CCID_{50}$	128	0.516	168	2.091	126	21.770	8516
10 000 $CCID_{50}$	179	1.234	841	3.334	407	12.121	-
Cytotoxic Conc.[b]	8345	53.461	4542	20.973	254	27.638	8590

[a] Each IC_{50} was the mean of two to four experiments
[b] The 50% cytotoxic concentration (CC_{50}) evaluated according to the neutral red uptake procedure

Table 2. Selectivity of antiviral action

Viral inoculation dose	Selectivity ratio (CC_{50}/IC_{50}) of:						
	HBB	Oxoglaucine	Ribavirin	Disoxaril	PTU-23	Arildone	S-7
100 $CCID_{50}$	189.66	137.08	39.16	13.64	5.08	2.83	3.46
1000 $CCID_{50}$	65.20	103.61	27.04	10.03	2.02	1.27	1.01
10 000 $CCID_{50}$	46.62	43.32	5.40	6.29	0.62	2.28	-

the antiviral properties of this compound. It seems likely that ribavirin does not have one universal mechanism of action, and inhibit different viruses in different ways (Parker, 2005; Graci and Cameron, 2006). Some authors have reported values of 10-100 μg/ml as minimal inhibitory concentrations for some rhinoviruses like types 1A, 2, 13 and 56 (Sidwell *et al.*, 1972; Huffman *et al.*, 1973). Our results for the effect of ribavirin on the replications of human rhinovirus 14 are very similar. The inhibitory concentration 50% varies between 100 and 800 μM depending on the virus inoculation dose. Compared to other compounds tested, ribavirin reveals a moderate anti-rhinoviral effect.

Disoxaril and arildone are WIN compounds which inhibit virus uncoating by direct insertion into the hydrophobic canyon within the VP1 capsid protein (Zeichhardt *et al*., 1987; Eggers and Rosenwirth, 1988). The effect of disoxaril on the replication of entero- and rhinoviruses is well known. Generally, the rhinoviruses are less sensitive to disoxaril than the enteroviruses (Otto *et al.*, 1985; Fox *et al.*, 1986). The data presented in our paper are coincident with the results reported by Otto (1985). Compared to HBB, oxoglaucine or ribavirin, disoxaril shows rather weak antirhinoviral effect. The antirhinoviral effect of arildone is not significant.

PTU-23 inhibits the synthesis of viral 37S RNA as a result of suppression of the synthesis of a viral protein with regulatory functions in the replicative cycle (Galabov, 1979; Galabov and Dmitrieva, 1983; Galabov *et al.*, 1983). In primary tests PTU-23 exhibited borderline activity against rhinovirus H-17 (Galabov *et al*., 1977). In our study the effect of this compound was tested against human rhinovirus 14. The effect observed was close to the results of the team of Galabov, and PTU-23 showed too weak effect on the replication of the tested virus.

S-7 is known to prevent uncoating by direct interaction with the enterovirus particle (Lonberg-Holm *et al.,* 1975). No activity of this compound against HRV-14 was registered in our study.

In conclusion, the aporphinoid alkaloid oxoglaucine reveals a well pronounced inhibitory effect on the replication of human rhinovirus 14. The IC_{50} evaluated according to the neutral red uptake procedure varies from 44 to 179 μM depending on the viral inoculation dose. From the data above it can be considered that oxoglaucine, possessing a selectivity index above 100, is a prospective anti-rhinoviral substance and a candidate for further preclinical and clinical trials.

Acknowledgements

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Corrigendum

Odisseev H. (2016) About pathogenesis of the vaccine mumps meningitis. Acta Microbiologica Bulgarica, 32 (2): 139-141.

The author wish to inform readers that the Figure 1 was omitted from the above published paper. The missing figure will not be published due to lack of permission from the author of the original paper.

The Editorial Board

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In memoriam

Annotation on the naming of the Laboratory of Microbiology at University Hospital "Tsaritsa Yoanna-ISUL" EAD after its founder, Prof. Dafina Hadzhidimova, MD, DMedSc, HonSc

Prof. Dafina Hadzhidimova was born on 24 January 1907 in Sofia. She graduated with flying colours from Sofia First Girls' High School and in 1926 left Bulgaria illegally for Moscow. Between 1926 and 1932, she graduated from the Moscow Medical Institute and did a course of postgraduate study at the Department of Microbiology. Her dissertation topic was Adsorption and Isolation of the Epidemic Typhus Virus. She worked as a microbiologist at the Institute of Nutrition in Ivanovo-Voznesensk and as a research fellow at the Institute of Microbiology and Epidemiology, and later at the Department of Microbiology of the Smolensk Medical Institute on the problems of epidemic typhus.

In the early days of the war, during the evacuation of the Chkalov Serum and Vaccine Institute to the Urals, she became head of the Uranbash branch, organizing and supervising the on-site production of anti-gas-gangrene sera for the front. There she experimented with new techniques for immunization of horses, which became the subject of her doctoral dissertation Bivalent Gas-Gangrene Sera, defended immediately after the war (1945). Dafina Hadzhidimova was offered a job at the Moscow Medical Institute, which she refused and returned to Bulgaria 19 years after leaving her homeland (1945).

Dafina Hadzhidimova started working as deputy director at the Institute for People's Health (NIZPB). She organized the department for production of sera and vaccines, and later laid the foundation of the Institute for Drug Control, present-day Bulgarian Drug Agency.

In 1945, the Central Institute for Standards and Control of Biological Preparations was established, re-named State Control Bacteriological Institute in 1949. Its director was Prof. Dr. Dafina Hadzhidimova.

The equipment and operation of the microbiological laboratory at the Department of Epidemiology and Infectious Diseases were her merit, as well as the publishing of scientific papers in the Bulletin of National Institute of Infectious and Parazititic Diseases, which still exists to this day.

Prof. Dafina Hadzhidimova founded the Scientific Society of epidemiology, microbiology, virology, immunology, parasitology, infectious diseases, which held the first national congresses and conferences.

In 1952, she was appointed to associate professorship in the newly founded Institute for Specialization and Post-Graduate Education of Doctors (ISUL), tasked with organising the Department of Microbiology.

She initiated joint discussions of clinicians and microbiologists on infectious complications in patients, and the sending of samples for examination with subsequent joint discussion of the therapeutic plan.

In addition to microbiology and virology, Prof. Hadzhidimova also worked for the advancement of immunology. Eminent Bulgarian scientists, then young doctors and assistant professors, who had been trained under Prof. Hadzhidimova and had also received specialized training abroad, began their professional careers there.

She also pioneered and promoted sanitary microbiology.

Prof. Hadzhidimova structured training and diagnostic laboratories, trained teaching staff, devised curricula. She published a Guidebook on Mi-

crobiological Diagnostics and Especially Dangerous Infections. She conducted scientific and experimental work, the training of postgraduate students, clinical interns, and the specialization of personnel in basic and thematic courses. Thus she paved the way and promoted postgraduate training and microbiology specialization examinations. She also created a vivarium for experimental purposes.

All professional fields in the hospital laboratories of microbiology and virology expanded, as well as the network of newly established health and anti-epidemic centres nationwide: microbiology, virology, epidemiology, food hygiene, water control, etc. Hers was the undeniable merit of creating specialists for the prophylaxis network in Bulgaria. This was the starting point for trained specialists at all levels - regional, municipal, clinical, before they went on to work across the country.

Prof. Hadzhidimova published Guidebook on Microbiological Diagnostics and Especially Dangerous Infections (co-authors D. D. Hadzhidimova, St. Grigorov); Guidebook on Microbiological Diagnostics (1964, co-authors D. D. Hadzhidimova, U. V. Tyagunenko, G. V. Mihailov); Microbiological Diagnostics, 1975.

She organized the microbiology specialization examinations and for many years was Deputy Rector of ISUL.

She initiated the appointment of two associate professorships of epidemiology and virology, subsequently establishing two separate departments with trained staffs and equipment.

In 1930 Prof. Hadzhidimova married her colleague Dr. Emeliyan Strokov, an epidemiologist, who died at the outset of the war of epidemic typhus. She lost two young children, a boy and a girl. She arrived in Bulgaria with her youngest son, Vova, Dr. Vladimir Em. Strokov, who survived the war, and later worked as Chief Assistant Professor at the Department of Microbiology.

Prof. Hadzhidimova was awarded the title "Honoured Scientist", and numerous medals and decorations.

The unification of the Department of Microbiology at ISUL with that of High Medical Insitute at the Medical Academy coincided with 65th anniversary before she retired.

Prof. Hadzhidimova passed away on 10 December 1984 at the age of 77.

This text is based on "*Memories of My and My Family's Past*" by Prof. D. Hadzhidimova

Rossitsa Vacheva

ACTA MICROBIOLOGICA BULGARICA Volume 32 / 4 (2016)

Cronicle

Former Institution with Traditions Who's Past Lives on in the Future

History of the Pharmacological Section at the Chemical Pharmaceutical Research Insitute (NIHFI)

The 1950s and 1960s saw a rapid growth of the chemical and pharmaceutical industry in Bulgaria. The Galenus factory in Sofia grew into a Chemical and pharmaceutical plant, with number of enterprises were established across the country: the Antibiotics plant in Razgrad, the Plant for veterinary medicinal preparations in Peshtera, cosmetics manufacturing plants in Sofia and Plovdiv, a plant in Troyan, a Chemical and pharmaceutical plant in Dupnitsa, etc. The manufacture of pharmaceuticals required full chemical, analytical and toxicological control evaluation. As laboratories of synthesis and phytochemistry developed new, original medicinal products were created, which prompted the need for speedy introduction of toxicological, pharmacological and clinical-pharmacological analysis. The centre of investigations became the Scientific Chemical and Pharmaceutical Research Institute (NIHFI), established simultaneously with the progression of this industry. The institute developed, keeping abreast with similar institutes in the countries of Central and Eastern Europe. Along with the laboratories of synthesis, biosynthesis, phytochemistry, analytical testing, organ preparations, a pharmacological unit was formed at the institute, which was later to become the Medico-biological devision. The initially created pharmacological unit of the early 1950s was headed by Al. Minchev, a graduate from the Department of Pharmacology at the Medical Academy, and by L. Daleva until it became Medico-biological devision, which was afterwards headed by M. Nikolova for 25 years (L. Daleva was Scientific Director), and in the last seven years by N. Tyutyulkova.

The purpose of the Medico-biological devision at NIHFI was to organize specialise laboratories and specialists for them for the purpose of conducting full screening and extensive pharmacological and toxicological characterization of synthesized chemical compounds and products derived from natural substances. The result of these efforts was the largest Medico-biological devision formed in Bulgaria in the 1970s and 1980s, with a staff of about 120, encompassing laboratories of pharmacology, toxicology, clinical pharmacology, pharmacokinetics, pharmacobiochemistry with an isotope laboratory, and laboratory of chemotherapy. Specialised laboratories were developed for studying the effects on the central nervous system (CNS) using EEG; the cardiovascular system; the vegetative nervous system; single organs; anti-inflammatory effect; cosmetics; dental products and toothpastes; X-ray laboratory; pharmacokinetic laboratory, pharmacobiochemical laboratory and laboratory of chemotherapy with microbiology and mycology laboratories; toxicology and pathomorphology laboratory. The availability of up-to-date equipment, including isotope and X-ray laboratories, and the skilled specialists opened the way to a wide range of modern evaluation methods of medicinal products at the large complex Medico-biological devision, at the time unique for Bulgaria. It thus became possible to carry out relatively large-scale screening of new compounds and extensive investigation of the substances with manifested effect.

The Medico-biological devision at the institute was the first to introduce systematic screening of novel substances - newly synthesised or newly isolated natural products – for neuropharmacological, cardiovascular and antispasmodic effect. Over the years, more than 5,000 original, newly synthesized and isolated natural products were subjected to trials.

Methods for the assessment of psychotropic, anti-hypoxic, anti-ischemic effects were introduced; an EEG method, methods for the assessment of the cerebral blood flow; analgesic, cardiovascular, gastrointestinal, dermatological, anti-inflammatory, antibacterial, antifungal effects. Specific methods were developed to assess the effects on neuromuscular conductivity, specific methods for evaluation the effects on blood clotting, capillary resistance, anti-atheromathosis agents, anti-inflam-

matory, etc. as well as histochemical methods.

A range of most advanced biochemical methods and approaches were adopted that helped clarify the pharmacodynamics of pharmaceutical drugs, such as: determination of the level of the biogenic amines noradrenaline, dopamine, and serotonin and their metabolites in different brain structures; radioisotope methods for determination of drug effects on specific receptors; investigation of the effect of certain drug groups on oxidative phosphorylation; determination of RNA metabolism in the brain, microsomal glycoprotein biosynthesis in the liver; studies on platelet aggregation, acetylcholinesterase activity, etc. The complexity of the Medico-biological section facilitated the implementation of all new biochemical methods on adequate pharmacological models, thus contributing to the objectivization of the results.

A significant share of the activities of the section involved comparative bioavailability studies (since 1967), the results of which were a obligatory part of the documentation for the registration of medicinal products. To determine the concentration of drugs and their metabolites in plasma and urine, modern methods, such as liquid chromatography and gas chromatography-mass spectrometry, were developed and adopted. Such studies were conducted on healthy volunteers at leading medical clinics with the authorization of the Committee for Medicinal Products.

Parallel studies were conducted on the possible toxicity – acute, sub-acute, or chronic toxicity, embryotoxicity, mutagenicity, carcinogenicity and local allergic and irritant action - using histomorphological methods.

The section was equipped with the largest vivarium in Bulgaria, with relevant breeding lines of mice, rats, rabbits, guinea pigs, hamsters, Beagle dogs, bred for experimental purposes.

Results were processed by applying contemporary statistical techniques.

All preliminary investigations were summarized at the Registration department, where a comprehensive dossier of the medicinal product was compiled, and afterwards passed on to the Clinical department for preparation for clinical trial authorisation.

Drug dossiers were submitted to the Committee for Medicinal Products for clinical investigation authorisation. The medicinal products applied in the clinical practice were both generic medicines, as well as original products created at the institute. By permission of the Committee for Medicinal Products, targeted, systemic clinical trials of Bulgarian products first began in 1962 in university clinics and hospitals across the country. The experts from the clinical department of the Medico-biological devision were involved in the development of the test methodology for a given medicinal product from a particular clinical group and directly participated in the study and analysis of the results. The cardiologists, neurologists, dermatologists and rheumatologists at the Department of Clinical Pharmacology of the Medico-biological section deserve special merit for the organization of the clinical part of the study.

The organization of experimental laboratories, the Registration and Clinical departments, and the qualification of the personnel followed the requirements for introducing new drugs to both domestic and foreign markets, especially the vast market of the USSR. Gradually, European and FDA requirements for approval of drugs were introduced.

The laboratories were headed by a multitude of highly trained professionals: doctors, dentists, veterinars, biologists, pharmacists, chemists, many of whom had specialised in renowned laboratories at home and abroad:

Doctors of Science, Senior Research Fellows (1st grade) (now full professors) with defended doctoral dissertations: Dr. L. Daleva, Dr. M. Nikolova, Dr. T. Harizanova, Dr. P. Manolov, Dr. R. Nikolov.

Doctors, Senior Research Fellows (2nd grade) with defended dissertations (now associate professors): Dr. N. Tyutyulkova, Dr. P. Arnaoudova, Dr. V. Chavdarova, Dr. J. Jordanov, Dr. N. Donev, V. Marinova (biologist), Dr. J. Illarionov, Dr. M. Taskov, Dr. Stefan Vankov, V. Ognyanova (chemist), Dr. V. Marinova, Dr. D. Stefanova, Dr. V. Dimova, A. Dryanska (chemist), Dr. S. Zarkova (vet. surgeon), Dr. O. Angelova, M. Dikova (MPharm), Dr. V. Atanasova, N. Ivanova (biologist), Dr. Z. Gendzhev (vet. surgeon).

Research Fellows: Dr. L. Petrova, St. Markova (biologist), Dr G. Tanev, Dr. O. Petkov, Dr. D. Delev (vet. surgeon), M. Deleva (biologist), Dr. R. Sheikova (stomatologist), Dr. Ivan Torlakov (vet. surgeon), Dr. P. Stefanova, Y. Gorancheva (biologist), Dr. M. Dencheva Dr. E. Kozhinkova, Dr. D. Hodzheva, Dr. S. Katsarova, I. Mihailova (chemist), R. Panikian (stomatologist), Galina Nakova (chemist), Nina Valchanova (biologist), Dr. Inna Kirkova, Dr. Julia Maslarova, S. Tuneva (biologist), Dr. Blenika Manolova, M. Vatsova (chemist), Dr. K. Bogoslovov (vet. surgeon), D. Bogoslovo-

va (MPharm), E. Kerimyan (MPharm), L. Tokuschieva (MPharm), S. Mandjukova (chemist), Julia Yaneva (biologist), Kina Konstantinova (chemist), Tsveta Potourlian (MPharm).

Specialists: L. Zankova (MPharm), Dr. G. Shumkov (vet.surgeon), St. Tsvetanov (chemist), Dr. E. Shumkova (stomatologist), Yanka Germanlieva (MPharm), Ralitsa Atanasova (MPharm), and a large number of laboratory assistants and technicians specialized in clinical laboratory, microbiology, pharmacology, zootechnicians, etc.

The specialists at NIHFI were initiators and active facilitators in organizing an extensive network of contracts with various scientific and clinical institutions for the full characterization of their medicinal products, where certain specific laboratories were created for that purpose.

Members of the Scientific Council of the Medico-biological devision were distinguished university and research experimental pharmacologists and clinicians who discussed and evaluated the results of the experimental and clinical trials characterising newly created medicines and their formulations. The Council also discussed the expediency of developing new drugs, new galenic formulations and drug combinations in order to meet the needs of clinical practice. NIHFI and the Medico-biological devision organized annual conferences and symposia to share and exchange experience with eminent specialists. For instance, a significant amount of experience was gained in the 1960s during the joint research on the possible toxicity of Metamizole – Pharmachim's analgin – with specialists from Hoehst, Germany, carried out by Bulgarian and Israeli clinical specialists. Undoubtedly, the Medico-biological devision at NIHFI contributed greatly to the inclusion of leading experts from all medical institutions in Bulgaria in the process of characterisation of the medicinal products offered by the manufacturing enterprises and Pharmachim institute, subsequently by Sopharma and Balkanpharma.

Some of the therapeutic products created at the institute, tested and introduced into practice by the Medico-biological unit were:

– Original: Nifimicin, Nivalin, Stenopril, Aescuvasin, Lonetil, Tabex, Glauvent, Tribestan and Combination Vitaton, Tempidon and its Combination Tempalgin, Dolyspan, Mukarthrin, Aligeron, Cratemon, Combinations of Piracetam - Phezam, Orocetam, Vitapiracen, Indovasin.

– Generic synthetic products: analgin, indomethacin, Feloran, acetaminophen - paracetamol now paramax - piroxicam, cimetidine, ranitidine, carsil, chlophazolin, its combination Chlophadon, captopril, nifedipine, nitrendipine, prazosin, izodinit, monizid, atenolol, Sydnopharm, antistenocardin, molsidomine, verapamil; Troxevasin, venoruton, Pyramem - cinnarizine, flunarizine, medazepam, alprazolam, vinpocetine, furantril, clenbuterol - revealed immunostimulatory effect - famotidine, ketotifen, clemastine, insulin preparations.

– Generic chemotherapeutics and antibiotics: 5-Nitrox, ciprofloxacin, gentamicin, cefalexin, ampicillin, amopen, cefamandole, cefazolin, doxycycline, tetracycline, tobramycin, tubocin.

The institute has ceased to exist since the 1990s.

Many of the well-established therapeutic products of the institute did not sink into oblivion; they are still manufactured and sold in Bulgaria and abroad, and have their role in health care. Numerous young professionals who have passed through the school of NIHFI now work in pharmaceutical enterprises, laboratories and foreign companies.

Milka Nikolova and **Nadejda Tyutyulkova**

FOOD-3 INTERNATIONAL CONFERENCE
"THE CHALLENGES FOR QUALITY
AND SAFETY ALONG THE FOOD CHAIN"
NEW BULGARIAN UNIVERSITY

SOFIA, BULGARIA
MARCH 23rd - 25th 2017

WELCOME LETTER

Dear colleagues,

It is my great pleasure and honor to welcome you to the FOOD-3 International Conference: "The challenges for quality and safety along the food chain" which will take place at 23-25 March 2017 in New Bulgarian University, Sofia, the capital of Bulgaria. The conference traditionally is held under the auspices of NBU Rector - Prof. Plamen Bochkov. NBU is highly honored to host FOOD conference again.

Co-organizers of the conference are Bulgarian Food Safety Agency (Executive director - Dr. Damian Iliev), Bulgarian Society for Microbiology - a FEMS member (President - Acad. Angel Galabov) and Institute of Microbiology at Bulgarian Academy of Sciences (Director – Corr. member Christo Naidenski). Eminent experts of the Scientific committee have gathered together to share their expertise and contribute to a high-level scientific forum. Conference will cover and shape out diverse topics in food science as food and beverages quality and safety, contamination, microbiological control, toxicology, allergens and additives, assessment and audit, food biotechnology, nanotechnology in food, labelling and packaging, prebiotics and probiotics, organic food and GMO. FOOD-3 Conference will bring together prominent scientists and young researchers from more than 20 countries across Europe. The goal of the conference will also be to encourage young scientists to present their results. Exchange of ideas, results, and fruitful discussions on different facets will give fertile ground for fostering future cooperation. Along with keynote international speakers, leading experts of the Bulgarian National Food Safety Agency and main food industry branches will present their expertise, promulgate and discuss current and future challenges as well as the hot topics in food science.

Join us in Sofia, share your expertise with the participants, get in touch with recent advances in the field, debate, update your professional network and contribute to advance FOOD-3 in high-level scientific forum.

We are sure you will enjoy Sofia and the conference.

Sofia, a compelling city which interlaces in a lovely way ancient charm of thousand years history and pulsating up-to-date exuberance - is waiting for you.

Galina Satchanska
Chairperson
Conference Organizing Committee

Acta Microbiologica Bulgarica

GUIDE FOR AUTHORS

The journal ***Acta Microbiologica Bulgarica*** is organ of the Bulgarian Society for Microbiology (Union of Scientists in Bulgaria). The journal is continuation of the edited till 1993 journal of the same name, cited in Index Medicus and Medline. The new edition is published two times per year.

Types of articles

The journal publishes editorials, original research works, research reports, reviews, short communications, letters to the editor, historical notes, etc from all areas of microbiology. The manuscripts should not represent research results which the authors have already published or submitted in other books or journals. The papers submitted for publication in *Acta Microbiologica Bulgarica* are peer-reviewed by two experts from the respective scientific field who remain anonymous to the authors.

Article structure

The papers are published in English, accompanied by a bilingual summary (English and Bulgarian). The papers should be typed with double-spacing (28-30 lines), on a white paper in A4 format, with margins of 3 cm.

The length of the original research paper, including the annexes (tables, figures, etc.) should not exceed a signature or printer's sheet (30,000 signs, that is, 16 pages with 30 lines each) in Times New Roman 12. The length of shorter reports should not exceed seven pages.The submitted manuscript must contain the name/s and surname/s of the author/s, the name and address of the institution and or organisation where it was prepared. The name and address of the corresponding author should be noted. The abstract should not exceed 250 words and should represent briefly the goals, methods, main results (with numerical data) and basic conclusions of the research.The most essential six key words must be added to the abstract. The manuscript contains the following sections: introduction, materials and methods, results, discussion, acknowledgements, references. The introduction must be concise with a clearly defined goal and with previous knowledge of the problem. The materials and methods ought to contain sufficient data to enable the reader to repeat the investigation without seeking additional information. The results should be presented briefly and clearly, and the discussion should explain the results.The measuring units and other technical data should be given according to the Sl-system. Illustrations (tables and figures) are submitted separately and their places in the text should be clearly indicated. Tables should be given in a separate sheet, numbered and above-entitled. The figures must be accompanied by legends in a separate sheet.

Reference style

The references used are cited in the manuscript as follows:

- In the case of single author - the author's surname and the year of publication (Petrov, 2012);
- In the case of two authors - the authors surnames and the year of publication (Petrov and Vassileva, 2013);
- In the case of more than two authors - the first author's surname, *at all*., and the year of publication (Christova *et al*., 2014).

The references included in the section "References" of the manuscript should be arranged first alphabetically and then further sorted chronologically if necessary. More then one reference from the same author(s) in the same year must be identified by the letters "a", "b", "c", etc. place after the year of publication.

Examples:
Reference to a journal publication:
Georgiev, P., V. Simeonov, T. Ivanov (2010). Production of thermostables enzymes. *Biotechnol. Biotec. Eq.* **27:** 231-238.
Reference to a book:
John, R., W. Villiam, G. Wilmington (2011). New approach for purification of enteroviral proteins, in: Peterson, K., A. Smith (Eds.), Methods in Proteinology. Elsevier, London, pp. 281-304.
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