

# 水体中克雷伯菌属的分布及其在水源性急性肠道感染中的价值

Rakhmanin YA\*, Ivanova LV, Artyemova TZ, Gipp EK, Zagainova AV, Maksimkina TN,

Krasnyak AV, Zhuravlyev PV, Aleshnya VV

俄罗斯联邦卫生部 A. N. Sysin 人类生态学和环境卫生学研究所, 俄罗斯 莫斯科 119991

**摘要:** 俄罗斯不同气候地区不同功能水体中克雷伯菌属广泛分布。克雷伯菌属可见于遭受生物、化学污染的集中供水的地表水源, 无防护的地下蓄水层, 缺乏有效清洁、消毒系统的饮用水。研究表明, 水体中的克雷伯菌属具有致病性和毒性, 对现代药物和消毒剂(氯、紫外线)具有抗性, 很容易穿透进入地下蓄水层。克雷伯菌属细菌有很强的致病性(粘附力、侵袭力、磷酸酯酶、卵磷脂酶、脱氧核糖核酸酶、溶血活性), 含有致病性遗传标记 *cnf-1*。克雷伯菌属(100 CFU/dm<sup>3</sup>)可引起急性肠道感染。在不检测总大肠菌群的情况下, 检测水体尤其是饮用水中的克雷伯菌属, 可以评估所用水的流行病学危险。

**关键词:** 克雷伯菌属; 流行病学危险; 抗药性; 致病因素; 水源性急性肠道感染暴发

**中图分类号:** R378 **文献标识码:** A **文章编号:** 1006-3110(2016)11-1400-10 **DOI:** 10.3969/j.issn.1006-3110.2016.11.035

## Prevalence of the genus *Klebsiella* in bodies of water and its significance in water-borne acute enteric infections

Rakhmanin YA\*, Ivanova LV, Artyemova TZ, Gipp EK, Zagainova AV, Maksimkina TN,

Krasnyak AV, Zhuravlyev PV, Aleshnya VV

A. N. Sysin Research Institute of Human Ecology and Environmental Health of the Ministry of Health of the Russian Federation, Moscow 119991 GSP-1, Russia

**Abstract:** High prevalence of *Klebsiella* species in water of water bodies in different climatic zones of Russia intended for multi-purpose use is established; in surface sources of centralized water supply depending on a degree of their biological and chemical contamination; in groundwater from unprotected aquifers; in drinking water that was insufficiently purified and disinfected. It is shown that *Klebsiella* spp. circulating in water preserve their properties as pathogens as well as virulence; they are resistant to modern drugs and disinfectants (chlorine, ultraviolet irradiation) and able to readily penetrate into aquifers. These strains of *Klebsiella* are known to have the significant pathogenic capacity (adhesive, invasive, phosphatase, lecithinase, DNAase, hemolytic activity) as well as genetic markers of pathogenicity *cnf-1*. The etiological role of *Klebsiella* species and their infective dose (100 CFU/dm<sup>3</sup>) causing acute enteric infections (AEI) are defined. Isolation of *Klebsiella* from water bodies and, especially from potable water in absence of total coliform bacteria (TCB) testifies for an insufficient role of the latter and presents an epidemic hazard in water use.

**Key words:** *Klebsiella*; Epidemic hazard; Resistance to antibiotics; Factors of pathogenicity; Outbreaks of waterborne AEI

## Introduction

Currently, significance of potentially - pathogenic bacteria of Enterobacteriaceae family in human infections is increasing<sup>[1-3]</sup>. The proportion of acute enteric infections (AEIs) caused by potentially - pathogenic enterobacteria (PPE), including *Klebsiella* species, in overall incidence of AEI by known etiology, by data of various

authors, varies from 12.8%-21.2% to 34.1%<sup>[1, 4-5]</sup>.

In 2011, genus *Klebsiella* of Enterobacteriaceae family got incorporated in the List of Microorganisms of the WHO Guidelines for Drinking-water Quality<sup>[6]</sup> since it is capable of causing waterborne enteric infections in high risk populations with weakened immune system, including the elderly and very little children, patients with burns or large open wounds, patients undergoing immunosuppressive therapy, individuals with acquired immunodeficiency syndrome.

**Correspondence:** info@sysin.ru

10 Pogodinskaya Street, Building 1, Moscow 119991 GSP-1, Russia.

Since PPE present normal transitory flora of the human and at the same time pathogens of infectious diseases, researchers' opinions concerning PPE as etiological agents of enteric infections are ambiguous. A number of authors<sup>[7-9]</sup> noted that assessing etiological significance of PPE in infectious processes required a statement regarding their pathogenic capacity that was underestimated in verification of acute enteric infections. This justifies the expedience of studying factors of pathogenicity in this group of bacteria both at phenotypic and molecular-genetic levels<sup>[9-11]</sup>. However, pathogenicity and regional peculiar features of acute enteric infections caused by PPE are not yet fully understood.

One of the most important aspects of the phenotypic characteristics of conditionally-pathogenic enterobacteria is their resistance to antimicrobial agents<sup>[10, 12-13]</sup>. As Bilev<sup>[14]</sup> indicated, massive proliferation of the antibiotic resistant strains in the populations of conditionally-pathogenic organisms became the most important challenge of clinical medication due to their high adaptive abilities.

With information on the role of potentially-pathogenic bacteria of Enterobacteriaceae family in human infections as a background, their circulation in water ecosystems with consideration of bacteria's high adaptive ability compared to classical infectious pathogens remains insufficiently investigated.

It is known that the aqueous environment may become a habitat for potentially-pathogenic organisms (PPOs) where they can survive for a long period of time and get accumulated. Survival and accumulation of microorganisms in water bodies are boosted by their persistence factors<sup>[9, 15]</sup>. Some authors noted that the water PPE strains possessed adhesive and hemolytic activities, enzymes like DNAase and hyaluronidase as well as resistance to antimicrobial agents<sup>[16]</sup>.

For confirming the timeliness of tackling the role of *Klebsiella* spp. as an epidemiological agent of waterborne AEI, it's necessary to gain the data on their counts in water bodies of different uses, their quantitative levels, antibiotic resistance, pathogenic factors and other biochemical properties, as well as links between AEI incidence in human and contamination of consumed water by *Klebsiella* species. Very few papers are devoted to addressing these issues and the data obtained are equivocal.

The goal of the present study is to summarize the findings of multi-year researches on the quality of various water bodies in different regions of Russia with isolation and quantitative analysis of *Klebsiella* counts, scrutinize biochemical properties of the isolated strains, identify the strains resistance to antimicrobials, assess their pathogenic capacity based on properties of pathogenicity and virulence, and confirm the significance of drinking water contaminated with *Klebsiella* spp. in AEI.

## Materials and methods

### Isolation of bacteria from water bodies

Water bodies were selected in various climatic zones in Russia like Middle Russia (the Moscow Region) and some areas with hot climate. They included water from surface-water basins like rivers and the Tsymlyansk water reservoir (including water intake locations for centralized water supply and recreation area supply), water from underground springs (boreholes and wells from protected and unprotected aquifers), potable water in the distribution network of water users' tap, drinking fountains, and wastewater at the stages of cleaning and disinfection.

Water samples were collected in accordance with the requirements of the State Standard<sup>[17]</sup>. In each sample, normalized bacteriological indicators were determined by Guidelines (Methodical Recommendations) MYK 4.2.1018-01<sup>[18]</sup> and MYK 4.2.1884-04<sup>[19]</sup>, which marked the level of bacterial contamination by total microbial count (TMC) per 1 cm<sup>3</sup> and amount of coliform organisms per 100 cm<sup>3</sup> by membrane filtration method. The samples were incubated on selective differential medium the ENDO. The grown bacteria were identified by oxydase test and lactose fermentation (for TCB, thermotolerant coliform bacteria (TtCB) and *E. coli*). Bacteria of the genus *Klebsiella* were isolated on selective solid mediums Endo and K2 followed by identification using test-systems "Enterotest-24" firms Lachema. When the water bodies in the Rostov Region were studied, most probable number (MPN) of *Klebsiella* in water was identified with the titration technique using the optimal accumulation medium and subsequent inoculation onto the K2 medium. High effectiveness of the K2 medium in relation to *Klebsiella* was assured by unlimited nutritive properties with incorporating the ingredients that were utilized mostly by *Klebsiella*

spp. (raffinose, urea). Gram-positive flora was eliminated with crystal violet and brilliant green<sup>[20]</sup>.

### Determination of biological properties of the strains isolated from water bodies

Resistance of the *Klebsiella* strains isolated from the water reservoir's and the river's water in the Rostov Region during 1996–2000 and 2006–2010 to 20 antimicrobials that were used in clinical practice during this study in given region as well as hemolytic activity and enzymes of pathogenicity (DNAase, lecithinase and phosphatase) were assessed.

Drug resistance was also studied in the *Klebsiella* strains isolated from water bodies in the Moscow Region to 14 broad-spectrum antimicrobial agents belonging to four classes of antibiotics most commonly used for treating enteric infections: penicillins (amoxicilin), cephalosporins (cefaperazone, cefepime, ceftazidime, ceftriaxone), aminoglycosides (amikacin, gentamycin 10 mcg and 120 mcg, netilmicin), fluoroquinolones (ciprofloxacin, moxifloxacin, levofloxacin) and carbapenems (meropenem, imipenemum). Assessing antibiotic resistance of the bacteria and interpreting the results were done in accordance with MYK 4.2.1890–04<sup>[21]</sup>. Factors of pathogenicity and virulence (adhesive, cytotoxic and invasive activity) of *Klebsiella* species were tested with both standard methods and modified assays using BGM cell culture and Hep-2<sup>[16, 22–25]</sup>.

The 'pathogenicity islands' of the Enterobacteriaceae family's strains were identified by polymerase chain reaction (PCR) in accordance with the manufacturer's instruction inserted into a set of reagents for DNA identification DNA-sorb-AM. The nucleotide sequences responsible for bacterial pathogenicity and virulence were identified by using four pairs of specially designed primers. PCR results were interpreted with use of electrophoretic separation of amplification products on agarose gel stained with ethidium bromide. For selecting nucleotide sequences and amplification reaction temperature, computer software Vector NTI Suite (US) was used.

### Establishment of the role of waterborne transmission in *Klebsiella* spread

Waterborne transmission of AEI caused by *Klebsiella*

spp. was confirmed in the organized group of children at a recreation center in hot climate. Local water supply relied upon the underground aquifer at a depth of 6 meters. Water was accumulated in the well where it was pumped out through ultraviolet lamps to the water tank and then to the distribution network consisting of the kitchen, drinking fountains and shower rooms. Quality of water in the water source and that consumed by children was assessed based on normalized bacteriological indicators. The *Klebsiellae* count was calculated on the K2 medium.

Studying significance of *Klebsiella* spp. in occurrence of AEI among residents of one of the settlements was carried out in Central Asia. Data on AEI incidence were submitted to the sanitary-epidemiological surveillance bodies that investigated every case by bacteriological testing of feces of each patient with detailed identification of pathogens. The water factor's role was confirmed with epidemiological methods and population surveys.

## Results

### Prevalence of *Klebsiella* spp. in surface and underground water sources

Assay of eight hundred water samples collected from water sources in the Moscow Region showed high prevalence of *Klebsiella* spp. In water bodies whose quality of water did not meet hygienic requirements for the TCB indicator, *Klebsiella* species were found in 19% of the samples and pathogenic *Salmonella* in 8%. Among TCB, amount of *Klebsiella* was higher than that of the other Enterobacteriaceae family's members. It was noteworthy that in groundwater of unprotected aquifers – wells, shell boreholes, springs located within the settlements' boundaries, *Klebsiella* spp. accounted for over 50% of TCB and in five areas, *Klebsiella* species were found in water with complete absence of coliform bacteria and *E. coli*. At the same time, *Klebsiella* count on the fomites of centralized water supply varied from 10 to 3,800 CFU per 100 cm<sup>3</sup>.

While studying water in surface-water reservoirs, we found high content of *Klebsiella* spp. and their wide spread across the whole aquatory of this water body. In the water reservoir in the Rostov Region as far as 180 km, *Klebsiella* count, on an average, amounted to 92,378 CFU/100 cm<sup>3</sup>, which outnumbered TCB (56,221 CFU/

100 cm<sup>3</sup>), and thermotolerant coliform bacteria (TtCB, 809 CFU /100 cm<sup>3</sup>), both reflecting disadvantaged epidemiological situation further confirmed by high *Salmonella* content of 75.50 MPN/1,000 cm<sup>3</sup>. The seasonality of *Klebsiella* amount was noted. They increased from the summer–autumn period (Table 1), which correlated with the dynamics of pathogenic enterobacteria, *Salmonella* ( $r=0.81$ ,  $P=0.002$ ).

Table 1 Sanitary–bacteriological characteristics of water in water reservoir in the Rostov Region in 2006–2010 (mean values)

Season	TCB (CFU/100 cm <sup>3</sup> )	TtCB (CFU/100 cm <sup>3</sup> )	<i>Klebsiella</i> (CFU/100 cm <sup>3</sup> )	<i>Salmonella</i> (MPN/1,000 cm <sup>3</sup> )
Spring	33,911	6,959	28,552	68.60
Summer	53,941	844	148,534	74.13
Autumn	80,809	887	150,049	83.90
Average	56,221	809	92,378	75.50

Data of the distribution of sanitary–indicative micro-organisms and *Klebsiella* in water of the water reservoir demonstrated that *Klebsiella* spp. along with TCB bacteria reflected the sanitary situation of a water body most precisely for they characterized the greatest bacterial contamination in the upper portion to where river water carrying pollution from the territory of several regions flowed. Contamination of a dam area was associated with towns and settlements located on the banks. The most polluted central portion pointed out to intensive processes of water self–purification as those that took place in regulated wetlands.

Testing of the quality of water in the river polluted with wastewater revealed that the mean *Klebsiella* count in this water body amounted to 204,099 CFU/100 cm<sup>3</sup>. Notably, analysis of the *Klebsiella* contamination’s degree along the river flow from the water reservoir to its mouth showed that the least polluted sites were cities’ water intake locations (*Klebsiella* count 22,808 CFU/100 cm<sup>3</sup> and 51,300 CFU/100 cm<sup>3</sup>). The most polluted portions within the river aquatory comprised those sites where urban wastewater was discharged, where the *Klebsiella* count amounted to hundreds of thousands CFU/100 cm<sup>3</sup>. In the river delta’s channels, mean concentration of *Klebsiella* was equal to 247,798 CFU/100 cm<sup>3</sup>. The same portions of the water reservoir were characterized by high epidemiological hazard, which got confirmed by the rate of isolating pathogenic *Salmonella* and their concentration, 385 MPN/1,000 cm<sup>3</sup>. Determination of correlation

between *Klebsiella* and *Salmonella* showed statistical significance ( $r=0.513$ ,  $P=0.0002$ ).

Prevalence of *Klebsiella* spp. in wastewater

It was found that household wastewater was one of the most significant sources of contamination of water body’s water with *Klebsiella* species that expressed more resistance in the processes of wastewater purification and outnumbered (126,000 CFU/100 cm<sup>3</sup>) all other indicative organisms at the water treatment plant’s outlet.

Table 2 Isolated bacteria at the purification stages in city’s sewage system in 2005–2006 (CFU/100 cm<sup>3</sup>)

Cleaning stages	TCB	TtCB	<i>Klebsiella</i>
Inlet chamber	132,000,000	66,000,000	78,000,000
Sedimentation tank with treatment modules	18,600,000	7,800,000	18,600,000
Brush load bioreactor	181,000	18,000	132,000
Cleaned water tunnel (discharge to the river)	72,000	7,260	126,000

Prevalence of *Klebsiella* spp. in drinking water

Because of such high prevalence of *Klebsiella* species in water of surface and underground sources, several instances of their detection in drinking water took place.

The system of running water treatment in city C did not have a typical set of facilities, where water underwent sedimentation and partial filtration followed by disinfection. That is why at water treatment stages (before chlorination), all assayed organisms were detected: TCB and *Klebsiella* in 100% of samples, TtCB in 76%, and *Salmonella* in 27%. At the distribution network’s outlet, water did not meet the hygienic requirements for TCB in 44.4% of samples and for TtCB in 14.8%. In meeting regulatory requirements for tap water fed into the distribution network, *Klebsiella* species were found in 33.3% of samples (Table 3).

Table 3 Sanitary–microbiological characteristics of drinking water in city C in 2006–2010 (average detection rate,%)

Year	Indicators regulated by the Sanitary Rules 2.1.4.1074–01		PPO *	Pathogenic organism
	TCB	TtCB	<i>Klebsiella</i>	<i>Salmonella</i>
2006	46.7	0.0	73.3	0.0
2007	34.6	0.0	69.5	0.0
2008	9.0	0.0	36.4	0.0
2009	6.8	0.0	3.4	
2010	73.0	13.0	80.0	12.4
Average	44.4	14.8	33.3	–

\* Note: Potentially–pathogenic organism

The study showed at city A water supply outlet, after its comprehensive treatment plant, water that did not

meet the hygienic requirements for TCB were detected in 8% of the samples, for TiCB in 1.3% and for *Klebsiella* in 13.3%. In the water supplied to the distribution network and meeting regulatory requirements, *Klebsiella* was detected in 7.2% of the samples. In water within the distribution network, the proportion of substandard samples in relation to TCB was as high as 28.3% . TiCB were detected in 10.5% of the samples and *Klebsiella* was reported in 37%. At the same time, *Klebsiella* was found in 16.6% of standard quality water samples (Table 4).

**Table 4** Sanitary-microbiological characteristics of drinking water in city A in 2006–2010 (average detection rate,%)

Year	Indicators regulated by Sanitary Rules 2.1.4.1074-01		PPO	Pathogenic organisms	
	TCB	TiCB	<i>Klebsiella</i>	<i>Salmonella</i>	Shigella
2006	26.0	17.0	45.0	0.0	0.0
2007	68.0	7.0	89.0	0.0	0.0
2008	48.5	24.7	51.5	10.3	1.0
2009	19.4	6.1	21.0	0.0	0.0
2010	19.8	6.8	17.8	0.0	0.0
Average	28.3	10.5	37.0	-	-

**Biological properties of *Klebsiella* pp. isolated from water bodies**

High prevalence of *Klebsiella* species in various water bodies determined the necessity for studying their biological and biochemical properties. Comparative assessment on antibiotic resistance, hemolytic, DNAase, cytotoxic, adhesive and invasive activity as well as other pathogenicity and virulence factors at the genotype level was done on 127 indicative potentially-pathogenic (*Salmonella*) and pathogenic enterobacteria isolated from water of various water sources in the Moscow Region. It was found that 60% of the assessed bacteria were resistant to amoxicillin (penicillins), 72% to moxifloxacin (fluoroquinolones), 68% to amikacin and 52% to ceftazidime (cephalosporins). In the setting of similar sampling (n=20) of indicative, PPE and pathogenic bacteria isolated from water of different water sources, *Klebsiella* spp. turned out to be 1.5 times more resistant to antimicrobials in use than indicative microorganisms (*Escherichia coli*) and 1.1 times more resistant than the pathogenic ones (*Salmonella*).

As chemical contamination of water reservoirs' water went up (concentration of copper, oil products and ni-

trites in water exceeds permissible limits), 40% of the PPE became resistant to 5–8 studied antibiotics. Correlation between *Klebsiella* drug resistance to broad spectrum antibiotics (3 to 4 generation) (n=28) and their virulence was found: bacteria resistant to two or more antimicrobial agents possessed more virulence or regained it up to 100% (*Klebsiella oxytoca*) as a result of situating themselves in the enabling conditions – being cultured in nutrient broth containing 0.5% glucose.

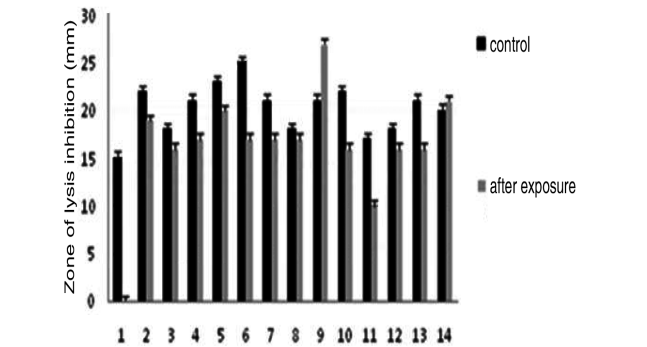
Adhesive activity of 45 strains of *Klebsiella* and *Salmonella* isolated from water of different water sources was assessed. Adhesive activity index per 100 BGM (buffalo green monkey) cells was within the range from 0.2 (strains with lower adhesion) to 19 (strains with higher-adhesion). 69.5% of the bacteria had high adhesion. All studied bacterial strains displayed high degree cytotoxicity and pathogenicity factors were assessed in the culture of transplanted cells. Virtually, all strains exhibited invasive activity except for *Klebsiella oxytoca*, whose invasive activity recovered only after 24 hours of incubation in nutrient broth containing 0.5% glucose.

PCR for detecting 'pathogenicity islands' in microorganisms showed that 24 PPE strains isolated from water bodies had 'pathogenicity islands' – specific DNA sequences associated with manifestation of pathogenic properties. The sequence encoding cytotoxic necrotizing factor –1 (cnf-1) has been found in 23/24 strains (except for *Klebsiella oxytoca*). The sequence associated with type-1 fibrin (fimA) and enterohemolysin (ehx) responsible for adhesive activity was present in all the 24 strains. The nucleotide sequence of iron regulatory protein-2 (irp-2) responsible for invasive activity was detected only in 10 strains of *Klebsiella* with invasive activity and was not found in 14 non-invasive strains including reference test organisms. Correlation between availability of the bacterial genetic material responsible for expression of pathogenicity and virulence (adhesive, invasive and cytotoxic activity) and manifestation of these properties was proven by the polymerase chain reaction (PCR) technique and *in vivo* method (bacterial contamination of the BGM cell line culture's passages).

In experiments, changes in biological activity of *Klebsiella* species due to influence of physical methods of water disinfection were found. Under the three-hour im-



part of the 4 Hz frequency magnetic field, *Klebsiella oxytoca* increased its resistance to 10 antibiotics by 20%. After the combined impact of ultraviolet (UV) irradiation (capacity of 254 nm–65 mW/cm2) and ultrasound field (ultrasonic wave frequency (22±1.65) kHz with capacity of each of the two emitters 0.1 kW), resistance of *Klebsiella pneumoniae* to 12 antibiotics increased by 30% (Fig. 1).



**Fig.1** Antimicrobial resistance of *Klebsiella pneumoniae* from water disinfected by a combination of UV light and ultrasound  
Legend: Axis X: 1 - amoxicillin/clavunate; 2 - levofloxacin; 3 - ceftazidime; 4 - amikacin; 5 - ciprofloxacin; 6 - cefepime; 7 - cefaperazone; 8 - gentamycin 10 mcg; 9 - gentamycin 120 mcg; 10 - ceftriaxon; 11 - moxifloxacin; 12 - netilmicin; 13 - meropenem; 14 - imipenemum.

It is found that *Klebsiella* spp. isolated from water of the water reservoir and the river in absence of significant differences between a numbers of circulating antibiotic resistant strains are most resistant to rifampicin, ampicillin and erythromycin (Table 5). As a result of studying on biological properties of *Klebsiella* spp., no significant differences between the numbers of antibiotic resistant strains circulating in the river *versus* the surface water reservoir in the Rostov Region were reported. Besides, *Klebsiella* spp. isolated from water of the water reservoir displayed resistance to tetracycline (81%) and chloramphenicol (79%), amoxicillin (84%) and azythromycin (78%). Their antibiotic resistance to polymyxin, streptomycin and neomycin was significantly lower (19.2% – 38.2%).

It has been found that *Klebsiella* species have a number of factors that, under certain circumstances, are able to exert pathogenic effects on the human body. Pathogenicity enzymes (DNAase, lecitinase and phosphatase) and hemolytic properties of *Klebsiella* species isolated from water bodies in the Rostov Region was studied in 212 strains of *Klebsiella pneumoniae*, of which 92 strains

were isolated from the water reservoir and 120 strains from the river. All of the strains had typical morphological, cultural and biochemical properties of given genus.

**Table 5** Antibiotic resistance of *Klebsiella* species isolated from water of the water reservoir and the river (%)

Antibiotics	Period of observation	<i>Klebsiella</i> species from water storage reservoir			<i>Klebsiella</i> species from river		
		Resistant	Moderately resistant	Sensitive	Resistant	Moderately resistant	Sensitive
Rifampicin	1996 – 2000	94.0	3.0	3.0	95.0	4.0	1.0
	2006 – 2010	88.7	6.3	5.0	86.0	7.7	6.3
Gentamycin	1996 – 2000	26.5	0.0	73.5	32.0	0.0	68.0
	2006 – 2010	22.0	0.0	78.0	19.0	0.0	81.0
Ampicillin	1996 – 2000	86.7	11.8	1.5	93.0	5.0	2.0
	2006 – 2010	84.0	13.0	3.0	81.2	12.5	6.3
Erythromycin	1996 – 2000	73.0	10.0	17.0	74.2	10.2	15.6
	2006 – 2010	77.0	11.0	12.0	77.3	10.4	12.3
Doxycycline	1996 – 2000	33.5	21.8	44.7	38.5	21.4	40.1
	2006 – 2010	37.7	24.0	38.3	42.2	16.5	31.3

As Table 6 shows, hemolytic activity of *Klebsiella* spp. isolated from water of the river significantly exceeded that of the same bacteria isolated from water of the water reservoir ( $t=3.67, P=0.001$ ), which was congruent with the higher level of biological and chemical contamination of water in the river. The high level of anthropogenic pollution of water bodies enhanced bacteria’s adaptive properties, in this case, pathogenicity factors. Hemolytic activity of *Klebsiella* was significantly higher in 2006–2010 than in 1996–2000 ( $t=3.24, P=0.02$ ), which was associated with a decline in industrial production back in 90s and accordingly reduction of the anthropogenic load on water bodies.

**Table 6** Activity of pathogenicity enzymes of *Klebsiella* species isolated from water of open–water areas

Water body	Period	Number of assessed cultures	Hemolytic properties (%)	Level of pathogenicity enzyme activity (%)		
				DNAase activity	Lecitinase activity	Phosphatase activity
Water reservoir	1996 – 2000	38	43.2	44.4	34.7	49.4
	2006 – 2010	56	56.5	55.7	46.7	57.1
River	1996 – 2000	54	67.8	53.8	48.2	64.3
	2006 – 2010	63	81.3	64.2	56.3	68.8

The number of *Klebsiella* species capable of manifesting their pathogenic properties was significantly higher in water of the river compared to that of the strains isolated from water of the water reservoir ( $t=2.444, P=0.035$ ). In water of the river, the proportion of *Klebsiella* spp. possessing lecitinase and phosphatase activity excee-

ded that in water of the water reservoir ( $t=2.794$ ,  $P=0.05$  and  $t=5.245$ ,  $P=0.006$ ). The count of *Klebsiella* spp with DNAase activity was also higher in water of the river. Among the assayed pathogenicity enzymes, phosphatase activity was most frequently detected, followed by DNAase and lecithinase activity. However, statistically significant differences in DNAase and phosphatase activity were not found among a number of strains. Correlations of the frequency of isolating *Klebsiella* spp. vs. lecithinase and DNAase with lecithinase and phosphatase activity ( $r=0.982$ ,  $P=0.02$  and  $r=0.960$ ,  $P=0.04$ ) were proven. Analysis of the occurrence of pathogenicity enzymes in *Klebsiella* spp. has demonstrated its increase in 2006–2010 compared to that in 1996–2000.

Thus, conducted studies have shown that the higher the level of water body's biological and chemical pollution (the river compared to the water reservoir) was, the more aggressive the studied bacteria became. Increase of the anthropotechnogenic impact on aquatic ecosystems (during the period from 2006 to 2010 compared to that from 1996 to 2000) has increased a number of bacteria containing pathogenicity enzymes, i. e. enhanced adaptive properties of the studied bacteria. Finally, presence of bacterial strains with pathogenicity factors in water was related to epidemic hazards of water use.

### Significance of *Klebsiella* spp. in etiology of enteric infections caused by water factors

In order to reveal correlation between the AEI incidence and the level of bacterial contamination of water used for drinking purposes, a disease outbreak in the organized group of children (8–12 years of age) on vacation down South was investigated.

The acute disease onset took place on day 10–12 after children's arrival, with a peak of cases occurring on day 3–4 after the first one. Almost in 50% of the infections, fever with body temperature of  $38^{\circ}\text{C}$ – $39.9^{\circ}\text{C}$ , headache, nausea, loose stools, dizziness and vomiting were observed. All patients complained of general fatigue and catharral phenomena in their pharynx. Duration of this disease was about 2–3 days, and fever persisted for 1–2 days.

To find the etiology of these mass acute enteric diseases, feces of 100 children and 15 employees were ex-

amined for *Klebsiella* spp. 77% of the patients recovered from diarrhea. Children were drinking a lot of water from water fountains.

It was found that the sources of aquifer's contamination were pit toilets of this village's individual sector with no sewage system located in immediate vicinity of the monitored area. Detection of just citrate-positive *Klebsiella* species in complete absence of *E. coli* in water of the water source illustrated the ability of *Klebsiella* spp. to penetrate into aquifers and survive in underground water and water-saturated soils for a long run.

The count of coliform bacteria in drinking water amounted to 26–55 CFU/100 cm<sup>3</sup>. Concentration of *Klebsiella* varied from 7 CFU/100 cm<sup>3</sup> to 20 CFU/100 cm<sup>3</sup>. By biochemical characteristics, vast majority of bacteria isolated from feces and water were identified as *Klebsiella pneumoniae*. Lack of ability of the *Klebsiella* strains isolated from water to fermentize lactose at  $44.5^{\circ}\text{C}$  should be noted since it was not typical of the clinical strains isolated from feces.

In serological identification, 17 out of 28 typed cultures had serovar K13 (over 70% of the strains isolated from water and 50% of the strains isolated from patients and exposed healthy individuals). Four patients had serovar K27. Assessing blood sera with autostrains and reference cultures showed the high antibody titer 1:32, which correlated with diagnostically significant titers of agglutinating sera for *Klebsiella* serotyping. Besides, cultures of serovars K8, K24 and K60 (sporadic strains) described as an AEI etiology agent were isolated from patients. There were no previous report on isolating serovar K27 in diarrheal diseases in literature.

In AEI outbreaks, the etiological roles of *Klebsiella* serovars K13 and K27 were confirmed with both isolating cultures of the similar serovar from patients, exposed individuals or from water used for drinking and presence of diagnostically significant antibody titers in the assessed blood sera obtained from patients. The data yielded demonstrated the pivotal role of the water transmission mode in occurrence of *Klebsiella* enteric infection in the tested population.

Examination of 100 individuals allowed establishing the minimum infective dose of *Klebsiella* causing AEI (from 70 to 200 CFU per 1 dm<sup>3</sup> of water) in drinking wa-

ter. The incidence got reduced to single case and eliminated at the *Klebsiella* concentration of 0–13 CFU per 1 dm<sup>3</sup> of water. With intensive water consumption, on average about a liter per person, speaking of the *Klebsiella* infective dose became feasible; it was about 100 cells that, once ingested, caused acute intestinal infections. The fact of disease occurrence at that level of *Klebsiella* in drinking water has been observed twice in different pediatric populations.

In the domain of studying etiological significance of PPE, including *Klebsiella*, as a cause of AEI, large-scale researches have been done over the course of 10 years in one of the settlements in Central Asia with the population of about ten thousand people. In each case of AEI, laboratory services identified an etiological agent according to the special algorithm. Shifts of leading pathogens in the overall AEI incidence were peculiar. Thus, the number of cases of dysentery has been steadily decreasing year by year. It got reduced by 3 times during 6 years with the stable number of enterocolitis caused by PPE, which proved significance of PPE in population's morbidity. Data on mortality of acute enteric infections among the city's population were also exemplary. If 5 individuals died of classical infections (dysentery, salmonellosis, typhoid B) during 6 years of observation, mortality associated with diagnosis of 'AEI of known etiology' caused by PPE amounted to 21 cases. Most lethal cases were reported in children less than one year of age.

As one of the examples of the role of *Klebsiella* spp. in AEI among city's residents, one may quote the data for one year when *Klebsiella* infections accounted for 58% of all AEI cases (261 cases) caused by PPE. *Klebsiella* infections reached their peak (over ten cases in a month) in the summer and autumn.

At the same time, assessment of sanitary-epidemiological quality of water intended for drinking and household purposes established the wide circulation of PPE in water; tap water supplied irregularly as scheduled and water from wells, hauzes, canals and irrigation ditches. Among PPE, *Klebsiella* species dominating in as far as a number of cases were concerned.

When the resistance to antibiotics of the strains isolated from water was studied, modified technique of membrane filtration was applied, which permitted not only to

establish a percentage ratio of strains resistant *vs.* susceptible to antibiotics but also to measure their amount in water depending on various types of water use. Thus in tap water, the number of *Klebsiella* strains resistant to ampicillin higher than 2 CFU per 100 cm<sup>3</sup> presented epidemic hazard in drinking water use. In water from surface water reservoirs intended for drinking purposes, the number of such bacteria was as high as several thousand CFU per 100 cm<sup>3</sup>. Mostly, those strains resistant to the penicillin group were isolated, while multiple drug resistant strains were circulating, predominantly, in surface water reservoirs. The majority of resistant *Klebsiella* strains were isolated from wells' water – up to 60 per 100 cm<sup>3</sup> with the increase in the summer and fall season, which coincided with the growth of a number of infections caused by *Klebsiella* during this period.

## Discussion

The data obtained showed that superior viability of potentially-pathogenic bacteria, *Klebsiella* species, was demonstrated both in monocultures and in microbial associations. In contrast, *E. coli*, which the sanitary-epidemiological safety assessment system of water use was based upon, turned out to be less resistant.

Increased viability of *Klebsiella* spp. in the water environment that was superior to that of indicative organisms' shelf life presented itself one of the factors making possible the detection of *Klebsiella* spp. in water sources and, once in a while, in drinking water that was considered to be safe based on the judgement by indirect indicators of biological contamination.

With consideration of the high count of *Salmonella* and *Klebsiella* at the water intake locations and their resistance to treatment with chlorine, complete inactivation of these organisms did not take place at cities' water treatment facilities. Cases of these bacteria's penetration into the water supply system have been reported. In the water distribution network's output, for instance, *Klebsiella* was found, on an average during 5 years of observation, in 56.5% of samples in City C and in 13.3% of samples in City A. Even if tap water met regulatory requirements for target indicators (TCB, *E. coli* and TiCB), it contained *Salmonella* (in two cases in City C) and *Klebsiella* (in 16.6% of samples in City A, in



26.6% in City C). In water of surface water reservoirs contaminated with wastewater, *Klebsiella* species were isolated from 100% of samples; in underground springs of unprotected aquifers, in about 50% of samples on an average<sup>[15]</sup>.

Other researchers have also demonstrated the high prevalence of *Klebsiella* in water bodies, including drinking water. In water of wells, boreholes, facilities for water storing in the City of Nyala in South Darfur (Sudan), *Klebsiella* was isolated from 15% of the samples<sup>[26]</sup>. In bottled water in the City of Katmandu (Nepal), *Klebsiella* was also found among coliform bacteria in 39.6%<sup>[27]</sup>. In water of the distribution network in Spain's settlements, members of eight genera of Enterobacteriaceae were found in absence of *E. coli*, among which *Klebsiella oxytoca* was the most frequent finding<sup>[28]</sup>. These data indicated the insufficient role of indicative TCB and *E. coli* in epidemic prevention and the expedience of reporting *Klebsiella* within the framework of water quality control. Significance of *Klebsiella* as a component of water control on the milk farms in the US was emphasized as well<sup>[29]</sup>.

The high *Klebsiella* count in water ecosystems with consideration of circulating drug resistant strains was consistent with potential hazards of water bodies. Research showed that *Klebsiella* spp. isolated from water were resistant to 33%, moderately resistant to 43% and susceptible to 24% of the utilized antibiotics.

Spread of the *Klebsiella* strains resistant to modern broad spectrum antimicrobial agents circulating through water bodies have been described in many papers<sup>[4, 10, 14-16, 26-34]</sup>. Antibiotic resistant strains of Gram-negative bacteria were isolated from wells, boreholes, surface water reservoirs in the rural areas of Nigeria. 19.3% of *Klebsiella* possessed a multidrug resistant trait<sup>[33]</sup>.

It is known that *Klebsiella* water strains have genetic markers of pathogenicity, which testifies their potential epidemic hazard. It has been confirmed in studies by Anganova and other authors<sup>[4-5, 8-9, 11, 22, 29]</sup>. Genetic markers of pathogenicity demonstrated the important steps of interactions between the pathogens and the cells of the macro-organisms (adhesion, hemolysin production and multiplication in the host tissues) thus displaying the etiological significance of PPE. The broadest set of genetic

markers was found in *Klebsiella* spp.<sup>[3]</sup>. In the area of anthropogenic pollution, enhancement of *Klebsiella* hemolysis activity, DNAase, lecithinase, and phosphatase, has been observed, which was emphasized in the papers of other authors as well<sup>[4]</sup>.

Within the framework of epidemiological investigations of enteric infections in the group of children, the etiological role of *Klebsiella* in pediatric diseases caused by intake of drinking water contaminated with *Klebsiella* spp. was proven. In other studies<sup>[2, 35]</sup>, it was argued that *Klebsiella* species were the major PPE representatives in AEI of known etiology.

## Conclusions

*Klebsiella* species are highly prevalent in water used for different purposes: in surface water reservoirs depending on the degree of their biological and chemical contamination, in groundwater of unprotected aquifers, in drinking water without enough efficient water treatment system.

*Klebsiella* species circulating in water intended for different types of water use preserve their pathogenicity and virulence properties. They have significant ability to penetrate into aquifers and are capable of prolonged survival in groundwater and resistant to modern drugs, which enables them to act as AEI pathogens.

Under real epidemiological circumstances, the etiological role of *Klebsiella* species along with their low infective dose in transmitting water-borne AEI is proven.

The indicative roles of conventional coliform indicators are insufficient for water quality control. *Klebsiella* content often exceeds the TCB counts in the sources of water supply and, with consideration of its high resistance to chlorine, *Klebsiella* is frequently found in drinking water in absence of TCB, which presents epidemic hazard in water use and determines the necessity for rectifying the *Klebsiella* minimum infective dose, incorporating given bacteriological indicator in a program of epidemiological monitoring and timely reporting within the framework of risk assessment in the context of water-borne infections associated with PPE.

## Acknowledgements

The authors of this article express their appreciation

to members of the WHO Working Group who included the detection of *Klebsiella* spp. into the WHO Guidelines for Drinking-water Quality (2011) based on the materials submitted by professionals from Russia. The special gratitude is addressed to Elena Frolova for her high professional translation of the article into English and her editing made as a microbiologist.

## Author Contributions

Rakhmanin YA and Ivanova LV designed the investigation, controlled all stages of its realization; Artyemova TZ, Gipp EK, Zagainova AV, Maksimkina TN and Krasnyak AV conducted microbiological analyses of water, analyzed the data and drafted the manuscript; Zhuravlyev PV and Aleshnya VV provided data on sanitary condition of surface water and drinking water in Rostov region. All authors participated in critical revision of the manuscript, read and approved the final manuscript.

## References

- [1] Bokova AG. Role of conditionally-pathogenic organisms in etiology of acute enteric infections and a problem of dysbacteriosis in children. Doctor of Med Sci Dissertation, 1991.
- [2] Bondrenko VM. Role of conditionally-pathogenic bacteria in chronic inflammatory processes at various sites. Publishing House 'Triada', 2008.
- [3] Gizatulina SS. Conditionally-pathogenic enterobacteria in AEIs in early childhood and improving their microbiological diagnosis. Abstract of the Cand of Med Sci Dissertation, 1989.
- [4] Anganova EV, Chemezova NN, Yermolaeva NV, et al. Characteristic of conditionally-pathogenic causative agents of acute enteric infections. Infect Pathol J, 2000, 17(102):12-13.
- [5] Mavzyutov AR, Fialkina SV, Bondarenko VM. 'Islands' of pathogenicity of conditionally-pathogenic enterobacteria. J Microbiol, 2002, 6:5-9.
- [6] WHO Guidelines for Drinking-Water Quality, 4th ed. Vol. 1: Recommendations. Geneva: World Health Organizations, 2011.
- [7] Bondrenko VM, Fialkina SV, Agapova OV. *Klebsiella* spp. and klebsielliosis. Publishing House 'Triada', 2008.
- [8] Bochkov IA, Lavrenova ES, Yurkov LP. Impact of therapeutic bacteriophages on conditionally-pathogenic and symbiotic flora of the gut. Epidemiol Infect Dis, 2009, 2:48-51.
- [9] Yegorova SA, Kaftyreva LA, Makarova MA. Pathogenic potential of *Klebsiella* spp. as pathogens of acute enteric infections. Herald of the Medical Military Academy, 2008, 2(22):535-536.
- [10] Anganova EV. Antibiotic resistance of conditionally-pathogenic enterobacteria isolated from patients with acute enteric infections. The Siberian Medical Journal, 2012, 7:98-99.
- [11] Anganova EV, Dukhanina AV, Savilov ED. *Klebsiella* species in etiological profile of bacterial AEIs, assessment of their pathogenicity at the level of phenol- and genotype. Epidemiology and Vaccination, 2011, 6(61):62.
- [12] Mikhailova LV. Biology of conditionally-pathogenic microorganisms causing acute enteric infections. Abstract of the Cand of Med Sci Dis-

- sertation, 2011.
- [13] Strachunsky LS. State of antibiotic resistance in Russia. Clinical Pharmacology, 2000, 2:6-9.
- [14] Bilev AE, Zhestkov AV, Abalkin ME. A way of overcoming drug resistance of conditionally-pathogenic bacteria and fungi. Materials of the All-Russian scientific-practical conference 'Actual issues of epidemiology at the contemporary stage', 2011: 63-64.
- [15] Zhuravlev PV, Aleshnya VV, Golovina SV, et al. Monitoring for bacterial contamination of water reservoirs in the Rostov Region. Hygiene and Sanitary, 2010, 5:33-35.
- [16] Zaiganova AV. Developing approaches to assessing risks of water-borne bacterial enteric infections' occurrence. Abstract of the Cand of Biol Sci Dissertation, 2010.
- [17] State Standard (GOST) 31942 - 2012 (ISO 19458:2006). Drinking water. Sampling for microbiology testing. 2012.
- [18] MYK 4.2.1.1018-01. Sanitary-microbiological analysis of drinking water. 2001: 42.
- [19] MYK 4.2.1884-04. Sanitary-microbiological and sanitary parasitological analysis of water from surface bodies of water. 2004: 75.
- [20] Methodical recommendations 'Detection and quantification of *Klebsiella* in target testing of environmental objects'. 2004: 8.
- [21] Guidelines 4.2.1890-04. Testing susceptibility of microorganisms to antimicrobial agents. 2004:68.
- [22] Bondrenko VM. Genetic markers of virulence in conditionally-pathogenic bacteria. Journal of Microbiology. Epidemiology and Immunobiology, 2011, 3:94-99.
- [23] Pert SJ. Fundamentals of culturing microorganisms and cells. 'Mir', 1978.
- [24] Elbert BY. Practical manual of medical microbiology and sanitary-bacteriological testing methods, 1957: 357.
- [25] Isberg RR. Determinants for thermophilic cell binding and plasmid-encoded cellular invasion detected in the absence of the pseudotuberculosis invasion protein. Ibio 57:1998-2005.
- [26] Abdelrahman AA, Eltahir YM. Bacteriological quality of drinking water in Nyala, South Darfur, Sudan. Environ Monit Assess, 2010.
- [27] Subedi M, Aryal M. Public perception about drinking jar water and its bacteriological analysis. Nepal Med Coll, 2010, 12(2):110-114.
- [28] Burgos Y, Beu L. Common origin of plasmid encoded alpha-hemolysin genes in *Escherichia coli*. BMC Microbiol, 2010, 10:193.
- [29] Zadoks RN, Griffiths HM, Munoz MA, et al. Sources of *Klebsiella* and *Raoultella* species on dairy farms: be careful where you walk. J Dairy Sci, 2010, 94(2): 1045-1051.
- [30] Anganova EV. Biological properties of conditionally-pathogenic bacteria of water ecosystems. Hygiene and Sanitary, 2010, 5(1):67-68.
- [31] Yezepchuk YV. Biomolecular basis of bacterial pathogenicity. Medicina, 1985: 240.
- [32] Blanch AR, Galofre B, Lucena F, et al. Characterization of bacterial coliform occurrences in different zones of a drinking water distribution system. J Appl Microbiol, 2007, 102(3):711-721.
- [33] Oluyeye JO, Dada AC, Odeyemi AT. Incidence of multiple antibiotic resistant Gram-negative bacteria isolated from surface and underground water sources in southwestern region of Nigeria. Water Sci Technol, 2009, 59(5):1929-1936.
- [34] Tenover FC. Global problem of antimicrobial resistance. Russ Med J, 2005:1-6.
- [35] Yegorova SA. Characteristic of *Klebsiella* species isolated in the setting of enteric dysbiosis. Abstract of the Cand of Med Sci Dissertation, 2007.