Co-existence of *Legionella* and other Gram-negative bacteria in potable water from various rural and urban sources

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**Abstract**

A total of 320 potable water samples were collected from various rural and urban sources located in the Lublin region of eastern Poland. They comprised: 55 samples of treated (chlorinated) tap water from rural dwellings distributed by the municipal water supply system (MWSS), 111 samples of treated tap water from urban dwellings distributed by the MWSS, 45 samples of untreated well water from household wells and 109 samples from private water supply systems (PWSS) distributing untreated well water. Water samples were examined for the presence and species composition of *Legionella*, *Yersinia*, Gram-negative bacteria belonging to family Enterobacteriaceae (GNB-E) and Gram-negative bacteria not belonging to the family Enterobacteriaceae (GNB-NE), by filtering through cellulose filters and culture on respectively GVPC, CIN, EMB and tryptic soya agar media. The occurrence of *Legionella* in the samples taken from the outlets of the urban MWSS was high (77.5%), and significantly greater compared to frequencies noted in rural MWSS (7.3%), and samples of well water from household wells (28.9%) and PWSS (13.8%) \((p < 0.001)\). Strains *L. pneumophila* serogroups 2-14, *L. pneumophila* serogroup 1 and *Legionella* spp. (species other than *L. pneumophila*) formed respectively 64.3%, 17.5%, and 18.2% of total isolates from urban MWSS, 100%, 0, and 0 of those from rural MWSS, 69.2%, 7.7%, and 23.1% of those from household wells, and 66.7%, 0, and 33.3% of those from PWSS. The concentration of *Legionella* strains in the positive samples from urban MWSS exceeded the threshold limit value of 100 cfu/100 ml in 86.1%, while in the other sources this value was not exceeded. No *Yersinia* strains were isolated from the examined water samples. Altogether 8 species or genera of Gram-negative bacteria belonging to Enterobacteriaceae family (GNB-E) and 10 species or genera of Gram-negative bacteria not belonging to the Enterobacteriaceae family (GNB-NE) were found in the examined samples. In the MWSS samples, an inverse relationship was found between *Legionella* and GNB-E and the numbers of *Enterobacter* spp. and *Serratia* spp. strains were significantly more common in the samples without *Legionella*. By contrast, in the PWSS samples, the numbers of *Enterobacter* spp., *Klebsiella* spp. and *Salmonella* spp. were distinctly and significantly greater \((p<0.01-p<0.001)\) in the samples containing *Legionella*. Among GNB-NE, *Pseudomonas aeruginosa* strains occurred significantly more frequently in samples containing *Legionella* (for MWSS and well water separately \(p<0.05\), for total samples \(p<0.001\)). Similarly, strains of *Flavobacterium breve* and *Xanthomonas* spp. occurred significantly more often in the samples with *Legionella*, while the numbers of *Aeromonas* spp. and *Vibrio* spp. strains were significantly greater in the samples not containing *Legionella*. In conclusion, a health risk could be associated with exposure to the water from urban MWSS because of the high prevalence and concentration of *Legionella*, and with exposure to well water from PWSS because of the correlation of occurrence of *Legionella* and potentially pathogenic Enterobacteriaceae strains, and the possibility of synergistic effects. The adverse effects could be also due to the significant correlation of *Legionella* and *Pseudomonas aeruginosa* that occurred in water from various sources.

**Key words**

Gram-negative bacteria, *Legionella*, Enterobacteriaceae, Non-Enterobacteriaceae, correlation, potable water, farms, wells, water supply systems

**INTRODUCTION**

*Legionella pneumophila* and related species are fastidious Gram-negative bacteria, developing mostly in natural and anthropogenic aquatic environments, which in humans may cause atypical pneumonia (*Legionellosis*, *legionnaires’ disease*) or flu-like illness [1-3]. People become infected with *Legionella* mostly by the inhalation of bacteria-laden aerosol droplets, less often by the oral route through drinking water and through traumatized skin or mucous membranes [3]. According to Stout et al. [4] potable water supplies that harbour *Legionella pneumophila* are an important source of community-acquired legionnaires’ disease.

The growth of *Legionella* in water depends on interaction with the other microorganisms such as protozoa, heterotrophic bacteria, green algae and cyanobacteria living in the same environment [5]. The most important relationships are: intracellular growth of *Legionella* in free-living amoebae and its growth and/or survival in biofilm matrices used as a shelter and source of nutrients [6]. A number of other microorganisms in biofilm, including heterotrophic bacteria, may promote the growth of *Legionella* by excreting extracellular compounds as carbon and energy sources [1, 2, 5, 7, 8]. Besides stimulatory effects, in the cases of some bacteria, inhibitory effects are also possible [5].

The aim of the present study, designed as a continuation of earlier research [3, 9], was to determine the degree of contamination with *Legionella* of potable water from various rural and urban sources, and to assess relationships between the presence of *Legionella* and other heterotrophic Gram-negative bacteria belonging and not belonging to the family Enterobacteriaceae.
MATERIALS AND METHODS

Samples of water. A total of 320 samples of water used as a potable water for consumption were taken in the years 2007-2010 during summer months (June-August) from various places located in the Lublin province of eastern Poland. Water samples were taken into sterile glass bottles with a volume of 700 ml at the following sites:

- 45 samples of well water were taken directly (with use of a pail) from 45 private household wells located on farms in 6 villages.
- 109 samples of well water were collected from taps of 109 private water supply systems (PWSS) conducting untreated and unheated water from household wells to outlets within farm buildings located in 11 villages.
- 55 samples were taken on 55 farms in 3 villages from cold-water taps of the municipal water supply system (MWSS) distributing treated (chlorinated) groundwater, pumped from a depth of 40-100 m. The taps were equipped with aerators or other endings for better outflow of water.
- 111 samples were collected in the years 2007-2010, during the period May-November, in the city of Lublin (eastern Poland), from hot-water taps of the municipal water supply system (MWSS) distributing treated (chlorinated) groundwater, pumped from a depth of 40-100 m. The taps were equipped with aerators or other endings for better outflow of water.

Processing of samples. Water samples were examined for the presence of the following Gram-negative bacteria (GNB): (a) Legionella; (b) Yersinia; (c) non-fastidious Gram-negative bacteria belonging to the Enterobacteriaceae family (GNB-E); (d) non-fastidious Gram-negative bacteria not belonging to the Enterobacteriaceae family (GNB-NE).

For the recovery of Legionella, water samples of 300 ml volume were filtered through cellulose filters (pores 0.45 μm, Millipore, USA). Filters were washed for 10 min in acid buffer (pH 2.2), then rinsed in Ringer solution (Merck, Darmstadt, Germany) and finally placed on isolation agar medium. For recovery of Yersinia, GNB-E, and GNB-NE, water samples of 100 ml volume each were filtered through cellulose filters (pores 0.45 μm, Millipore, USA), and finally placed on the appropriate isolation agar medium.

Isolation and identification of Legionella strains. The buffered charcoal yeast extract (BCYE) agar medium supplemented with the Growth Supplement SR 110 A and the Selective GVPC Supplement SR 152 E (Oxoid, Basingstoke, Hampshire, England) [10-13] was used for isolation of Legionella (further referred to as GVPC medium). Inoculated agar plates were incubated for 7 days at 37°C with an everyday check of growth. Colonies of Gram-negative bacteria grown on agar plates were incubated for 7 days at 37°C. Isolates suspected to be Legionella were identified with the microtest API Systems 20E (bioMérieux, Marcy l’Etoile, France).

Isolation and identification of GNB-E. The eosin methylene blue (EMB) agar (Merck, Darmstadt, Germany) was used for isolation of bacteria of the Enterobacteriaceae family. Inoculated agar plates were incubated for 24 hrs at 37°C. The grown colonies were counted and differentiated and the isolates identified to species or genus level with the microtest API Systems 20E (bioMérieux, Marcy l’Etoile, France).

Isolation and identification of GNB-NE. The tryptic soy agar (bioMérieux, Marcy l’Etoile, France) was used for isolation of bacteria not belonging to the Enterobacteriaceae family. Inoculated agar plates were incubated for 24 hrs at 37°C. The grown colonies were counted and differentiated and the isolates identified to species or genus level with the microtest API Systems NE (bioMérieux, Marcy l’Etoile, France).

Statistical analysis. The data were analysed by Student’s t-test. The value p<0.05 was considered significant.

RESULTS

Prevalence and concentration of Legionella in potable water from various sources. The occurrence of Legionella in the samples taken from the outlets of the urban municipal water supply system (MWSS) was high (77.5%) and significantly greater compared to the occurrence noted in the rural MWSS, and in the samples of well water from household wells and from the private water supply system (PWSS) (p<0.001) (Tab. 1). In total samples, the most common were isolates belonging to the Legionella pneumophila serogroups 2-14, found in 81 samples (73.0%) from urban MWSS, in 4 samples (7.3%) from rural MWSS, in 9 samples (20.0%) from household wells, and in 10 samples (9.2%) from the PWSS distributing well water. Isolates belonging to Legionella pneumophila serogroup 1 were found in 22 samples (19.8%) from urban MWSS and in one sample (2.2%) from household wells. Strains classified as Legionella spp. (species other than L. pneumophila) were found in 23 (20.7%) samples from urban MWSS, in 3 samples (6.7%) from household wells and in 5 samples (4.6%) from PWSS. With regard to only positive samples, strains L. pneumophila of serogroups 2-14, L. pneumophila serogroup 1 and Legionella spp. formed respectively 64.3%, 17.5%, and 18.2% of total isolates from urban MWSS, 100%, 0, and 0 of those from rural MWSS, 69.2%, 7.7%, and 23.1% of those from household wells, and 66.7%, 0, and 33.3% of those from PWSS (Tab. 1).
Regarding the concentration of Legionella strains in water, the most adverse situation was noted in urban MWSS, where in only 13.9% of positive samples it was below the threshold limit value of 100 cfu/100 ml [14], while in 52.4% it was within the range 100-200 cfu/100 ml, and in 33.7% of samples it was above 200 cfu/100 ml. By contrast, in all samples of water taken from rural MWSS, household wells and PWSS, the concentration of Legionella was below 100 cfu/100 ml (Tab 1).

**Correlation of Legionella with other Gram-negative species.** No Yersinia strains were isolated from the examined water samples. Altogether, 8 species or genera of Gram-negative bacteria belonging to the Enterobacteriaceae family (GNB-E) were identified. Enterobacter spp., Klebsiella spp. and Serratia spp. were isolated most often (Tab. 2). The numbers of GNB-E isolates from well water (household wells, PWSS) were significantly greater compared to isolates from MWSS (101 vs. 24 strains, p<0.001). No GNB-E strains were isolated from urban MWSS (Tab. 2). Interesting relationships between the prevalence of Legionella and GNB-E were found. While in MWSS samples an inverse relationship was found between these 2 groups and the numbers of Entero bacter spp. and Serratia spp. strains were significantly more common in the samples without Legionella, in the PWSS samples the numbers of Entero bacter spp., Klebsiella spp. and Salmonella spp. were distinctly and significantly greater (p<0.01-p<0.001) in the samples containing Legionella (Tab. 2).

Table 1. Occurrence of Legionella in various sources of potable water

<table>
<thead>
<tr>
<th>Source of potable water</th>
<th>L. pneum. 1</th>
<th>L. pneum. 2-14</th>
<th>L. pneum. 1 + L. pneum. 2-14</th>
<th>Leg. spp.</th>
<th>L. pneum. 1 + Leg. spp.</th>
<th>L. pneum. 2-14 + Leg. spp.</th>
<th>Total positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Municipal water supply systems</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urban</td>
<td>N=111</td>
<td>0</td>
<td>52 (46.9%)</td>
<td>11 (9.9%)</td>
<td>5 (4.5%)</td>
<td>7 (6.3%)</td>
<td>86 (77.5%)a</td>
</tr>
<tr>
<td>Rural</td>
<td>N=55</td>
<td>0</td>
<td>4 (7.3%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4 (7.3%)b</td>
</tr>
<tr>
<td>Well water</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Household wells</td>
<td>N=45</td>
<td>1 (2.2%)</td>
<td>9 (20.0%)</td>
<td>0</td>
<td>3 (6.7%)</td>
<td>0</td>
<td>13 (28.9%)a</td>
</tr>
<tr>
<td>Private supply systems</td>
<td>N=109</td>
<td>0</td>
<td>10 (9.2%)</td>
<td>0</td>
<td>5 (4.6%)</td>
<td>0</td>
<td>15 (13.8%)b</td>
</tr>
<tr>
<td>Total</td>
<td>N=320</td>
<td>1 (0.3%)</td>
<td>75 (23.4%)</td>
<td>11 (3.5%)</td>
<td>13 (4.0%)</td>
<td>7 (2.2%)</td>
<td>118 (36.9%)</td>
</tr>
</tbody>
</table>

Explanation: *p<0.05, **p<0.01, +++p<0.001. + prevalence of bacterial species significantly greater in the samples without Legionella, ++p<0.01, ++++ prevalence of bacterial species significantly greater in the samples with Legionella, +p<0.05, ++p<0.01, ++++p<0.001.

Table 2. Co-existence of Legionella with Gram-negative bacteria belonging to Enterobacteriaceae family in various sources of potable water

<table>
<thead>
<tr>
<th>Source</th>
<th>Municipal water supply systems</th>
<th>Well water</th>
<th>Total water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urban</td>
<td>Rural</td>
<td>Total</td>
</tr>
<tr>
<td>Citrobacter spp.</td>
<td>Leg + N=86</td>
<td>Leg (-) N=25</td>
<td>Leg + N=51</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>1 (25.0%)</td>
</tr>
<tr>
<td></td>
<td>Escherichia coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Enterobacter spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Haemophilus spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Klebsiella spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Pantoea spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Salmonella spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Serratia spp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Explanation: Leg + = Legionella present; Leg (-) = Legionella absent; N=number of samples; in individual fields numbers of positive samples and frequencies of occurrence are given; *p<0.05, **p<0.01, +++p<0.001. prevalence of bacterial species significantly greater in the samples without Legionella, +p<0.05, ++p<0.01, ++++p<0.001. prevalence of bacterial species significantly greater in the samples with Legionella, +p<0.05, ++p<0.01, ++++p<0.001.
DISCUSSION

The prevalence and concentration of Legionella in the outlets of the urban municipal water supply system (MWSS) distributing hot, chlorinated water, was significantly greater compared to the examined rural water sources. The levels of Legionella exceeded the Polish threshold limit value of 100 cfu/100 ml in 66.7% of total samples (86.1% positive) [14]. The risk is enhanced by the fact that in 19.8% of the samples there occurred the most virulent strains of Legionella pneumophila serogroup 1. The presented results represent a greater hazard compared to the samples of tap water from urban MWSS examined in our earlier work [3], which showed a Legionella prevalence lower by over 25%, and did not contain the strains examined in our earlier work [3], which showed a Legionella prevalence lower by over 25%, and did not contain the strains of Legionella pneumophila serogroup 1. It is possible that the presence of aerators or other endings in the taps or other outlets was more common in this study and thus favoured proliferation of legionellae.

The prevalence of Legionella in the outlets of the rural MWSS recorded in the present study was very low, which could be partly explained by the fact that the system distributed only cold water. It was significantly lower compared to our earlier work, while the prevalence of Legionella in well water from household wells and private water supply systems (PWSS) was similar [3].

Yersinia, the other fastidious Gram-negative bacterium transmitted by water, was not found in the present study in the well and MWSS water samples, in contrast to earlier Polish papers reporting the isolation of Yersinia strains from well water in a rural environment [11, 15].

The prevalence of Gram-negative bacteria belonging to the family Enterobacteriaceae (GNB-E) in well water samples was high and similar to that of Gram-negative bacteria not belonging to the family Enterobacteriaceae (GNB-NE). By contrast, in water samples from the outlets of urban MWSS, the GNB-E were absent, and in the samples from rural MWSS occurred with a low frequency. Most probably this it was due to the great vulnerability to chlorination, and imperfect adaptation to persist and proliferate in specific ecological niches of MWSS, such as aerated taps.

It is noteworthy that the prevalence of some potentially pathogenic GNB-E strains (Salmonella spp., Klebsiella spp., Enterobacter spp.) was especially high in well water from a private water supply system (PWSS) and showed a highly significant correlation with Legionella. This phenomenon, which creates a potential risk to people drinking well water from PWSS, may be due to the distribution of not-chlorinated

![Table 3. Co-existence of Legionella with Gram-negative bacteria not belonging to Enterobacteriaceae family in various sources of potable water](image-url)
water, and possibly also to the composition and construction of the PWSS forming a specific niche favourable for both groups of pathogens.

In contrast to GNB-E, the isolation frequency of Gram-negative bacteria not belonging to the Enterobacteriaceae (GBN-NE) in water samples from urban and rural sources was similar. Among them, the most common were strains of Flavobacterium breve and Vibrio spp. which were smaller from the samples containing Legionella. Our results are in accordance with those obtained by Moritz et al. [7] and Murga et al. [8] who found an integration of Legionella pneumophila and Pseudomonas aeruginosa in biofilms, and with those obtained by Cotuk et al. [5] who found experimentally that the cultures of Pseudomonas stimulated the growth of L. pneumophila, while the cultures of Aeromonas inhibited this growth. The correlation between Flavobacterium breve and Legionella stated by us, was earlier reported by Wadowsky and Yee [16], who noticed a satellite growth of Legionella pneumophila around colonies of Flavobacterium breve. In general, our results support the views of other authors who have reported the stimulative effects of Pseudomonas and Flavobacterium strains on growth and/or survival of Legionella [1-3, 5, 7, 8, 17-19]. These results could well be explained by the fact that both Pseudomonadaceae and Legionella grow well in similar aerobic niches constituted by water aerators. Stojek [20] found a much greater isolation frequency of Gram-negative bacteria in water from indoor taps equipped with aerators or other endings than in outdoor taps without these gadgets (80.0% vs. 44.4%).

By contrast, the presented study does not confirm the inverse correlation between Legionella and Pseudomonas aeruginosa in shower water samples that was reported by Leoni et al. [21], or the suppression of growth of Legionella by Pseudomonas aeruginosa reported by Kimura et al. [22].

CONCLUSIONS

In conclusion, a health risk could be associated with exposure to water from the urban MWSS because of the high prevalence and concentration of Legionella, and with exposure to well water from PWSS because of the correlation of occurrence of Legionella and potentially pathogenic Enterobacteriaceae strains and the possibility of synergistic effects. The adverse effects could be also due to the significant correlation of Legionella and Pseudomonas aeruginosa that occurs in water from various sources.

REFERENCES