

Original article

Microbial content of drinking water in Finnish and Russian Karelia – implications for atopy prevalence

Background and aim: The influence of microbial quality of drinking water from different sources on the occurrence of atopy has been poorly examined. This study was undertaken to clarify the association between the overall microbial content in drinking water and the occurrence of atopy among schoolchildren from two neighbouring areas with profound differences in living conditions and lifestyles.

Methods: Drinking water samples were obtained from kitchens of nine schools in North Karelia, Finland and of nine schools from Pitkäranta, the Republic of Karelia, Russia. The pupils of these schools were participants of the Karelian Allergy Study. Occurrence of atopy, determined by skin prick test positivity (one or more) to 14 common airborne and food allergens, was measured in all 563 children, aged 7–16 years, from these 18 schools. Water samples were analysed using standard methods for drinking water analyses including viable counts for *Escherichia coli*, intestinal enterococci, coliform bacteria and heterotrophic bacteria. In addition, total cell counts including both viable and nonviable bacteria, algae and protozoans were assessed using epifluorescence microscope with 4'-6-diamidino-2-phenylindole (DAPI) staining.

Results: In Finland, 29% of the children were sensitized to birch when compared with 2% of the Russian children ($P < 0.0001$). Overall, sensitization rates for any of the pollens were 39% and 8% ($P < 0.0001$), and for any of the allergens 48% and 16%, respectively ($P < 0.0001$). Because of substantial differences in raw water sources and treatment practices, the total numbers of microbial cells in drinking water were many-fold higher in Russia than in Finland. A dose–response relationship was found for occurrence of atopy and the DAPI value indicative of microbial cell content in the water ($P < 0.0001$). Further, multivariate logistic regression analysis revealed that high ($> 10^6$ cells/ml) and intermediate (10^5 – 10^6 cells/ml) DAPI values were associated with reduced risk of atopy (odds ratio 0.34, 95% confidence interval 0.20–0.57 and 0.39, 0.23–0.69, respectively), independently from other factors.

Conclusion: High overall content of micro-organisms in drinking water may be associated with reduced risk of atopy, independently from other determinants.

L. von Hertzen¹, T. Laatikainen²,
T. Pitkänen³, T. Vlasoff⁴, M. J. Mäkelä¹,
E. Vartiainen², T. Haahtela¹

¹Skin and Allergy Hospital, Helsinki University Central Hospital, Helsinki, Finland; ²Department of Health Promotion and Chronic Disease Prevention, National Public Health Institute, Helsinki, Finland; ³Department of Environmental Health, National Public Health Institute, Kuopio, Finland; ⁴The North Karelian Center for Public Health, Joensuu, Finland

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Dr Leena von Hertzen
Skin and Allergy Hospital
Helsinki University Central Hospital
PO Box 160
00029 HUCH
Helsinki
Finland

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Substantial disparities in atopy prevalence among schoolchildren were recently found between Finnish and Russian Karelia, irrespective of the geographical proximity of the study areas and the similarity of climatic and vegetative conditions (1). However, due to the wide economic gap between the areas, the living conditions are different, and resemble in Russian Karelia those seen in Finland circa 50 years ago.

Common to a traditional lifestyle and farm environment, both consistently associated with reduced risk of atopy (2, 3), is the heavy exposure to saprophytes in soil

and vegetation. This exposure to saprophytes may be one crucial factor in the induction of the regulatory network involved in the development and maintenance of immunological homeostasis of the gut and respiratory tract mucosa (4–6). Micro-organisms in this respect need not to be viable, as even nonviable microbial components are immunobiologically active (7–8).

Previous data from Europe have shown that consumption of untreated farm milk in early life may, independently from other determinants, confer protection against asthma and hay fever at school age, and this effect was considered to be mediated through exposure to microbial components in such milk (9). Concordant data have been obtained from schoolchildren in Grete (10), New Zealand (11) and England (12). It is therefore reasonable to

Abbreviations: DAPI, microscopic analysis of total cell counts with specific DNA staining; LPS, lipopolysaccharide; TLR, Toll-like receptor.

assume that consumption of untreated water in childhood might similarly confer protection against atopy and atopic disease. This issue has been poorly investigated; one study from Ethiopia found an inverse association between consumption of river water, as contrasted with piped water and atopic eczema, (13) and another study from Latin America showed a weak inverse association between consumption of untreated river water and atopy (14). No data from Europe are available as consumption of untreated surface water in developed world is very uncommon. The Karelian Allergy Study thus provides a unique setting to assess the relationship between the microbial content in drinking water and the occurrence of atopy in two boreal areas.

Given the importance of oral route in inducing mucosal tolerance (6) and the ability of even nonviable microbial components to be biologically active (8), we set out to test the hypothesis that consumption of drinking water with high microbial content, no matter if viable or nonviable, in childhood might be associated with reduced risk of atopy.

Material and methods

Subjects

This study was a part of the Karelian Allergy Study carried out among schoolchildren and their mothers in eastern Finland and in western Russia to clarify the occurrence of atopy and atopic disease and their risk factors on both sides of the border (1). Here, the association between microbial cell counts in drinking water obtained from school kitchens and occurrence of atopy in children of those schools is reported. In Finland, a representative sample (with respect to geographical location and size) of nine out of the 22 municipal schools in the area were chosen, whereas in Russia, all the nine such schools in the area were included. The children, aged 7–16 years, were the pupils of these 18 schools. Subject and living characteristics are presented in Table 1.

Skin prick testing

Skin prick tests against 14 common aero- and food allergens, birch, timothy grass, mugwort, cat, dog, cow, horse, house dust mite, fish, cow's milk, wheat, egg, peanut, were performed in all children, and additionally, hazel nut to a subgroup of children, as described in detail elsewhere (1). A positive (histaminedihydrochloride) and negative (solvent) control were used throughout the study. A wheal diameter ≥ 3 mm at least to one allergen was considered to be indicative of atopy.

Analysis of microbial cell counts in waters

All water samples were collected from the kitchens of the schools. The membrane filtration methods were used for the determination of viable counts of *Escherichia coli*, coliform bacteria and intestinal enterococci. In *E. coli* and coliform bacteria analyses, the Finnish national standard method SFS 3016 (15) with LES Endo Agar (Merck KgaA, Darmstadt, Germany) was used. The counts of intestinal enterococci were analysed according to the international standard ISO 7899-2 (16), and the heterotrophic plate counts after aerobic incubation for 3 days at 22°C were determined as stated in

Table 1. Subject and living characteristics and sensitization rates to allergens among the Finnish and Russian children

	Finnish	Russian	P-value
<i>n</i>	128	435	
Mean age (SD)	11.6 (2.5)	11.3 (2.5)	0.3225
Males, <i>n</i> (%)	72 (56.3)	213 (49.0)	0.1473
Cat <1 year, <i>n</i> (%)	16 (12.5)	156 (35.9)	<0.0001
Dog <1 year	23 (18.0)	100 (23.0)	0.2270
Parental farming, <i>n</i> (%)			
<1 year	8 (6.3)	86 (20.0)	0.0003
Currently	7 (5.5)	71 (16.4)	0.0016
Sensitization <i>n</i> (%)			
Birch pollen	37 (28.9)	8 (1.8)	<0.0001
Any pollen*	50 (39.1)	35 (8.1)	<0.0001
Any aeroallergen†	61 (47.7)	69 (15.9)	<0.0001
Any allergen‡	61 (47.7)	69 (15.9)	<0.0001

* Birch, timothy grass, mugwort.

† Birch, timothy grass, mugwort, cat, dog, cow, horse, house dust mite.

‡ Birch, timothy grass, mugwort, cat, dog, cow, horse, house dust mite, wheat, fish, cow's milk, egg, peanut, hazel nut.

the international standard ISO 6222 (17). Before direct microbial cell counts, the samples were preserved by adding 37% formaldehyde to obtain a final concentration of 2%. One millilitre of the surface water samples and 20 ml of the groundwater samples were filtered through a black 0.22- μ m pore size nuclepore membrane filter (Whatman, Clifton, NJ, USA) and stained with a DNA stain 4'-6-diamidino-2-phenylindole (DAPI) (18). The DAPI direct counts were enumerated with an epifluorescence microscope (Olympus BH-2, Tokyo, Japan) equipped with an ocular grid. Each child was linked with the DAPI value of his/her school.

Statistical analyses

Data were analysed using the chi-square test for categorical data and *t*-test for continuous data. The Mann-Whitney *U*-test was used for heterotrophic plate counts and total cell counts (DAPI). In assessing the relationship between atopy and DAPI values, a three-grade categorization, based on the distribution of the values, was performed: DAPI high, $> 10^6$ cells/ml; DAPI intermediate, 10^5 – 10^6 cells/ml and DAPI low, $< 10^5$ cells/ml. Multivariate logistic regression analysis was used to identify factors independently associated with the occurrence of atopy. Factors included in the model were chosen on the basis of our earlier results from the whole population (1) and comprised age, gender, exposure to cat allergen <1 year of age, parental farming <1 year of age and currently and the three DAPI categories. A two-tailed *P*-value of < 0.05 was considered statistically significant. The analyses were performed using the SAS System for Windows V8 (SAS Institute Inc., Cary, NC, USA) and the SPSS 12.0.1 (SPSS Inc., Chicago, IL, USA) for Windows software.

Results

Source and treatment of the waters

Except two schools, which used water from their own wells, all other seven schools in Russia used surface water from the lake Ladoga or associated rivers. No chemical treatment of these raw waters had been performed, with the exception of two cases, in which the water had been heavily

chlorinated in a pulp factory. By contrast, all Finnish schools used ground water alkalized in municipal water works, and five out of the nine waters had additionally been treated with ultraviolet disinfection before distribution.

Microbial content of the waters

Indicators of faecal contamination, *E. coli* and intestinal enterococci, were found in samples of three out of the nine schools in Russia, whereas in none of the schools in Finland, and coliform bacteria (faecal and nonfaecal) in six out of the nine schools in Russia, and none of the schools in Finland ($P = 0.009$). The mean number of viable heterotrophic bacteria in Russia was 29 100 colony forming units (CFU)/ml, whereas the respective count in Finland was only 6 CFU/ml ($P = 0.000$). As to the microscopically assessed total cell numbers (DAPI), a ninefold higher mean value in Russia as compared with Finland was obtained; 1 858 000 vs 204 600 cells/ml, respectively ($P = 0.001$) (Table 2).

Occurrence of atopy and protective factors

Atopy was substantially less common in Russia than in Finland. Sensitization rate for birch was 28.9% in Finland and 1.8% in Russia ($P < 0.0001$), and any of the pollens (birch, timothy grass, mugwort) 39.1% and 8.1% ($P < 0.0001$), respectively. Overall, sensitization rates to any of the allergens tested were 47.7% in Finland and 15.9% in Russia ($P < 0.0001$) (Table 1).

When occurrence of atopy was stratified according to the three categories (high, intermediate and low) of DAPI values, a dose-response relationship was obtained ($P < 0.0001$) (Table 3).

Multivariate regression analysis to identify factors associated with the occurrence of atopy revealed that high DAPI values were strongly associated with reduced

Table 2. Microbiological quality of drinking water in Finnish ($n = 9$) and Russian ($n = 9$) schools

	Finnish	Russian	P-value
Total coliforms/100 ml			
Mean (SD)	0	6 (10)	0.009*
Range	0–27		
<i>Escherichia coli</i> /100 ml			
Mean (SD)	0	3 (6)	
Range	0–16		
Enterococci/100 ml			
Mean (SD)	0	1 (1)	
Range	0–3		
Heterotrophic viable bacteria/ml			
Mean (SD)	6 (12)	29 118 (86 581)	<0.0001**
Range	0–38	5–260 000	
Total cell count (DAPI)/ml			
Mean	204 643	1 856 004	0.001†
Range	28 540–1 021 000	76 040–8 879 000	

* Chi-squared test for the difference in occurrence of coliforms.

† Mann-Whitney *U*-test for the difference in bacterial counts.

Table 3. Occurrence of atopy (skin prick test positivity to one or more allergens) according to DAPI categories (total cell counts/ml) among Finnish and Russian Karelian children

	DAPI*			P-value
	Low	Intermediate	High	
Atopy†, <i>n</i> (%)	43 (41.1)	39 (21.8)	48 (17.1)	<0.0001

* DAPI low, <10⁵ cells/ml; intermediate, 10⁵–10⁶ cells/ml; high, >10⁶ cells/ml. Each child had been linked with the DAPI value of his/her school.

† Atopy = at least one positive skin prick test result.

Table 4. Multivariate regression analysis to identify factors associated with atopy among the Karelian children

	OR (95% CI)	P-value
Age	1.03 (0.95–1.12)	0.480
Sex; males vs females	1.68 (1.10–2.57)	0.017
Cat <1 year	0.39 (0.23–0.66)	0.0005
Parental farming <1 year	0.43 (0.18–1.03)	0.0567
DAPI		
High >10 ⁶ cells/ml	0.34 (0.20–0.57)	0.004
Intermediate 10 ⁵ –10 ⁶ cells/ml	0.39 (0.23–0.69)	0.093
Low <10 ⁵ cells/ml	ref. = 1	

risk of atopy (0.34, 0.20–0.57, $P = 0.004$). In addition, intermediate DAPI values were also shown to be inversely associated with atopy (0.39, 0.23–0.69), although the P -value did not reach significance, probably because of the limited power of the analysis. Further, having a cat in early life was similarly inversely associated with atopy (0.39, 0.23–0.66, $P = 0.0005$), and parental farming in early life showed a weak inverse relationship with atopy (0.43, 0.18–1.03, $P = 0.057$) (Table 4).

Discussion

We found in this study that high microbial content in drinking water was inversely associated with atopy in a dose-dependent manner and independently from other determinants. The high content of micro-organisms in drinking water may be a surrogate marker for some other, yet unidentified factors associated with the poor living conditions in Russian Karelia, or, equally possibly, can exert direct immunomodulatory potential when consumed.

The emerging picture indicates an essential role for environmental saprophytes in stimulating the innate immunity and the regulatory network (5, 19). The ability of the innate immunity, specifically Toll-like receptors (TLR), to recognize commensal bacterial products has been found to have a crucial role in mammalian physiology; stimulation of TLRs by commensals appears to be involved in the maintenance of mucosal epithelial homeostasis and protection against epithelial injury (4).

Although we assessed the quality of drinking water obtained from schools, not from homes of the children, there are good reasons to believe that the water quality in

schools well reflects that in the neighbouring area generally. Lake Ladoga is the source of drinking water for most of the people in Russian Karelia, and water treatment plants similar to western countries are not found in that area (20). In Finland, by contrast, municipal water plants provide drinking water both for schools and homes in most cases.

Two earlier studies from the Tropics have found that occurrence of atopy or atopic eczema is lower in subjects consuming river water as contrasted with those consuming treated piped water (13, 14), in line with the present study. Albeit we presented here mere associations, not causality, the possibility that microbe-rich drinking water has immunomodulatory capacity cannot be excluded. As exposure to microbes in drinking water occurs repeatedly on a daily basis throughout the life, it is likely that, besides exposure to micro-organisms in soil and vegetation, it plays an important role in the maintenance of immunological balance of the gut and respiratory tract mucosa in Russian Karelia. Other factors that showed an inverse association with atopy here included cat ownership in early life and, to a lesser extent, parental farming in early life, in line with the previous results reported by us and others (1–3).

Microbiota in soil and natural surface waters appear to be associated with each other. Most recently, a Swedish study investigated the influence of bacteria imported from the surrounding soil (drainage area) on the bacterial communities in six boreal lakes in areas where the geoclimatic and vegetative conditions are virtually similar to those in Finland and Russia in the present study (21). It was found that bacterial communities in the lakes were to a high degree influenced by bacterial import from soil, even during a low flow period (21). Saprophytes in soil and vegetation gain easy access to the gut via drinking water in circumstances where surface water is used with minimal treatment, if any, as is the case in Russian Karelia.

Actinobacteria, which have been found to predominate in soil microbiota (22), represent also one of the most abundant groups of freshwater bacteria (23) and are ubiquitous in lakes of various types, size or geographical location (24, 25). A recent German–Russian study revealed that Actinobacteria can account for up to 63% of the bacterioplankton biomass (24). The phylum Actinobacteria comprises endotoxin-lacking bacteria including genera such as *Mycobacterium* spp., *Streptomyces* spp., *Actinomyces* spp., *Corynebacterium* spp. and *Bifidobacterium* spp. (26). Analyses of chemical markers of microbial communities in Finnish and Russian waters showed that the ratio of muramic acid (the major cell wall structure of particularly Gram-positive bacteria) to 3-OH fatty acids [the structure of lipopolysaccharide (LPS) and gram-negative bacteria] was roughly 3 : 1 both in Russian surface waters and in Finnish ground waters; however, the mean concentrations (ng/ml) for both markers were approximately 40-fold higher in Russia (data not shown).

The composition of microbial communities in drinking water may have significant implications for its immunomodulatory potential. Even heating of untreated water is unlikely to affect its immunobiological properties largely, as the major bacterial cell wall components, such as LPS, teichoic and lipoteichoic acids and muramic acid, are heat resistant (M. Salkinoja-Salonen, personal communication). Indeed, studies have shown that heat-killed bacteria have immunobiological properties similar to live bacteria (27).

Toll-like receptor 2 is the main recognition molecule for Gram-positive bacteria and their cell wall components except peptidoglycan fragments, which appear to be recognized by intracellular nucleotide-binding oligomerization domain (NOD)1 and NOD2 receptors (28–30). Toll-like receptor 4, by contrast, is the principal receptor molecule for endotoxin (LPS) in Gram-negative bacteria (28). Toll receptors involved in fungal recognition are less clear-cut, but both TLR2 and TLR4 seem to be involved (31). In addition to bacteria, fungi, protozoans and algae are abundant in fresh waters; however, their immunomodulatory potential is currently unknown.

Polymorphism in TLR2 gene has been shown to confer protection against atopy in farmers' children (32, 33), underscoring the significance of TLR2, the receptor for Gram-positive bacteria (28) in a microbe-rich environment. In a murine model, triggering of TLR2, more than of TLR4 or TLR7, was shown to induce the production of interleukin-10, the key regulatory cytokine, by dendritic cells (34). Interaction of TLR2 (but not TLR4 or TLR9) with its ligands was involved in T reg cell proliferation (35) and, interestingly, in inhibition of allergen-specific T helper 2 responses in sensitized individuals *in vitro* (36). It remains to be seen whether this TLR2 is the major player in the induction of regulatory network and mucosal tolerance.

Living in built, urban environments seems to be associated with increased risk of atopy as the natural connection of man and soil, existed since ancient times, has been severely disrupted during the last 50 years in affluent western societies (3). Natural waters are clearly a significant part of the whole issue of exposure to environmental saprophytes.

In conclusion, consumption of drinking water with high overall microbial cell content appeared to be inversely associated with the occurrence of atopy among schoolchildren.

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