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Serological findings and health complaints in exhibitors working on the 1999 Westfriese Flora in Bovenkarspel

A study following the outbreak of Legionnaires' Disease in visitors of the Westfriese Flora 1999

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Abstract

The aim of this study was to contribute to finding the source of a epidemic of Legionnaire's disease (LD) in visitors of the Westfriese Flora 1999 (West-Frisian Flower-show). The Westfriese Flora is a yearly flower exhibition combined with an agricultural and consumer products fair. The study targeted all persons without LD who had been working on the Westfriese Flora as volunteer, employee to the exhibition-organization or exhibitor. These persons were asked to fill in a questionnaire on their health status before and after the exhibition and their whereabouts during the exhibition. They were also asked to have a paired blood-sample taken to be tested for antibodies against *L. pneumophila*.
No clear relations between health complaints and specific places or other factors, like drinking tap-water, were seen. However, antibody levels were clearly increased in those working in hall 3, but not in those working at other places and therefore the main LD spreading source should be sought in hall 3.

The exact location, however, can not be inferred from this study with any certainty. From the two potential sources in hall 3 (a whirlpoolspa and a bubblemat) our data seem to indicate the whirlpoolspa as the more likely source, but this is not conclusive.

A further conclusion is that exposure to L. pneumophila seems to cause a slight elevation of antibody titers in those who do not develop LD, but that this increase is not accompanied by a demonstrable amount of milder forms of disease (apart from LD).

Contents

Samenvatting

In 1999 werd in de tweede week van maart een epidemie van veteranenziekte ontdekt, die tot nu toe de grootste is die ooit in Nederland werd waargenomen. Deze epidemie trad op bij bezoekers en medewerkers van de WestFriese Flora in Bovenkarspel. De WestFriese Flora is een jaarlijkse bloemententoonstelling, waarvan ook een consumentenbeurs en een landbouwbeurs deel uitmaken.

Dit onderzoek is opgezet om een bijdrage te leveren aan het vinden van de bron van deze epidemie. De onderzoekspopulatie bestond uit alle standmedewerkers op de Flora, de medewerkers van de Flora-organisatie en vrijwilligers die op de Flora aanwezig waren, maar die geen veteranenziekte hadden ontwikkeld. Deze personen kregen een vragenlijst toegestuurd met vragen over hun gezondheid voor en na de Flora, en hun verblijf (duur en plaats) op de Flora. Zij werden ook gevraagd tweemaal een bloedmonster te laten nemen voor onderzoek naar antilichamen tegen *L. pneumophila* en om een urinemonster op te sturen voor het bepalen van Lp1 antigeen in urine.

Om de vraag naar de plek van de mogelijke bron te beantwoorden werd gekeken of er een verband bestaat tussen:
1. De concentratie antilichamen tegen *L. pneumophila* in het bloed en specifieke plekken waar een persoon werkte of specifiek risicogedrag zoals het drinken van kraanwater op de Flora.
2. Gezondheidsklachten in de periode tijdens of vlak na het verblijf op de Flora en specifieke plekken waar een persoon werkte of specifiek risicogedrag

Er werd geen duidelijk verband gevonden tussen gezondheidsklachten en het werken op specifieke plaatsen of andere factoren. Ook de urine antigeen test was negatief voor alle deelnemers, zodat er geen aanwijzingen zijn dat de onderzoekspopulatie nog onontdekte personen met veteranenziekte omvat.
De hoeveelheid antilichamen tegen *L. pneumophila* in bloed was duidelijk hoger in personen die werkten in hal 3, waar een deel van de consumentenbeurs was gehuisvest. Er waren geen verschillen in de concentratie van antilichamen binnen de groep personen die op andere plekken werkten. Daarom kan hieruit worden geconcludeerd dat de belangrijkste bron van *L. pneumophila* zich in hal 3 moet hebben bevonden. Met behulp van ruimtelijke smoothing technieken kon de hoogte van de concentraties antilichamen worden weergegeven op de plattegrond van de consumentenbeurs. Daaruit kon de exacte locatie van de bron binnen hal 3 echter niet worden afgeleid. Van de twee mogelijke bronnen in hal 3 lijkt op grond van de antilichamenverdeling de whirlpool in hal 3 een iets aannemelijker bron dan het bad met een bubbelmat in hal 3, maar het verschil is niet overtuigend (zie tabel 10). Vanuit meer algemene kennis over de groeigewoonte van *L. pneumophila* en over de aërosolvorming kan echter

worden gezegd dat de whirlpool een vele malen waarschijnlijker bron is dan het bad met de bubbelmat. Dit wordt bevestigd door het bemonsteringsonderzoek, waarbij in hal 3 uitsluitend *L. pneumophila* werd aangetroffen in de whirlpool. Bovendien was deze whirlpool duidelijk sterker verontreinigd met *L. pneumophila* dan de apparaten uit andere hallen waarin *L. pneumophila* werd aangetroffen. Ook werden beide genotypen van *L. pneumophila* die bij de patiënten met veteranenziekten voorkwamen ook in deze whirlpool gevonden (zie RIVM rapport 213690003). Daarom kan worden geconcludeerd dat aerosolen afkomstig van deze whirlpool de belangrijkste bron van de epidemie zijn geweest.

In het rapport worden verder de kansen beschreven die standmedewerkers hadden om veteranenziekte te krijgen (attack rate), alsmede hun kans om verhoogde antilichamen tegen *Legionella pneumophila* te hebben zonder veteranenziekte te ontwikkelen, en de kans om gezondheidsklachten te ontwikkelen die verband zouden kunnen houden met besmetting met *Legionella.*
De kans op veteranenziekte voor standmedewerkers die niet in hal 3 werkten was 0,2%, vergelijkbaar met de kans die bezoekers hadden om veteranenziekte te krijgen (voor bezoekdagen na 22 februari bedroeg deze tussen de 0,1% en 0,5%). De kans voor standmedewerkers die in hal 3 werkten was echter ongeveer 10 maal zo hoog, namelijk 2,8%. De kans was 4,3% voor standmedewerkers die binnen 15 meter van de whirlpool in hal 3 werkten.

De kans om verhoogde antilichamen te hebben tegen *Legionella pneumophila* zonder veteranenziekte te hebben doorgemaakt was 26% voor standmedewerkers in hal 3 en minder dan 10% voor standmedewerkers die elders werkten. In bloedmonsters van een steekproef uit de algemene bevolking was dit 3%.
Het voorkomen van gezondheidsklachten tijdens of vlak na de Flora die lijken op de symptomen van legionellosis verschilde niet aantoonbaar tussen standmedewerkers uit hal 3 en standmedewerkers die elders hadden gewerkt.
Er werd eveneens geen verband gevonden tussen dergelijke gezondheidsklachten en de hoeveelheid antilichamen tegen *L. pneumophila* in het bloed. Wel was er hier en daar een verband tussen een individuele gezondheidsklacht en hetzij het werken in hal 3, dan wel de aanwezigheid van een hogere concentratie van antistoffen in het bloed.

Summary

In the second week of March 1999, the largest outbreak of Legionnaire's Disease (LD) in the Netherlands until now was discovered in patients who had been visiting or working on the West-Frisian Flower-show (Westfriese Flora) in Bovenkarspel. The Westfriese Flora is a yearly flower exhibition combined with an agricultural and consumer products fair.

The aim of this study was to contribute to finding the source of the epidemic. It targeted all persons without LD who had been working on the West-Frisian Flower-show as volunteer, employee to the exhibition-organization or exhibitor. These persons were asked to fill in a questionnaire on their health status before and after the exhibition and their whereabouts during the exhibition. They were also asked to have a paired blood-sample taken to be tested for antibodies against *L. pneumophila* and to send a urine sample for detection of urinary Lp1 antigen.
In order to find the source we looked at whether there is:

1. a relation between antibody titers of subjects and specific places they have worked, or other "risk" behavior like drinking water.
2. a relation between health complaints of subjects and specific places they have worked, or other "risk" behavior.

Urinary Lp1 antigen was absent in all participants, thus confirming that no undetected cases of LD were present. No clear relation between health complaints and specific places or other factors were seen. However, antibody levels were clearly increased in those working in hall 3, but not in other places and therefore the main LD spreading source should be sought in hall 3.

The exact location, however, can not be inferred from this study with any certainty. From the two potential sources in hall 3 (a whirlpoolspa and a bubblemat) our data seem to indicate the whirlpoolspa (see table 10) slightly stronger, but this is not conclusive. From the general knowledge on optimal growing conditions of *L. pneumophila* and on aerosol-spreading potential of devices, the whirlpoolspa is by far the most likely source of *L. pneumophila* containing aerosols in this hall. This is confirmed by the environmental investigation, in which this whirlpool was the only device in hall 3 found to be contaminated with *L. pneumophila*, while it was also more contaminated than contaminated devices found in other halls. Moreover, both genotypes of *L. pneumophila* cultured from LD-patients were grown from samples from this whirlpool (RIVM-report 213690003). Therefore it can be concluded that aerosols from the whirlpool in hall 3 are the main source of this epidemic of LD.

In this report we further describe the attack rates of Legionnaire's Disease (LD) in exhibitors, of being a serological case of infection with *Legionella pneumophila* (without having LD) and of health complaints resembling legionellosis (without having LD).

The attack rate of LD in exhibitors not working in hall 3 is 0.2%, similar to the attack rate in visitors (which after Februari 22nd varied from 0.1% to 0.5%), while that of exhibitors in hall 3 is more than 10 times as large (2.8%), while the attack rate in those working within 15 meters of the whirlpoolspa in hall 3 was 4.3%.
The attack rate for probable serological cases (defined both based on IgG and IgM) is 26% in hall 3 and less than 10% in the other halls, against 3% in a reference sample from the general population. No differences in health complaints resembling legionellosis (without having LD) could be ascertained between those working in hall 3 and those working elsewhere. No relations between such health complaints and antibody titers were seen. Some associations were seen between individual health complaints and either working in hall 3 or the height of antibody titers in serum.

1. Introduction

In the second week of March 1999, an exceptionally large number of patients with pneumonia were admitted to the Westfriese Gasthuis-hospital in Hoorn. This led to the discovery of the largest outbreak of Legionnaire's Disease (LD) in the Netherlands until now. An exploratory case-control study indicated that all case-patients had visited the West-Frisian Flower-show (Westfriese Flora) in Bovenkarspel (community Stede Broec) while only a minority of neighborhood controls had done so. The West-Frisian Flower-show is a yearly flower exhibition combined with an agricultural and consumer products fair, and was visited in 1999 by approximately 80.000 persons.

The National Institute of Public Health and Environment (RIVM) was contacted to investigate the source of the outbreak. This was done by :

- Keeping a national case-register to monitor the epidemic together with LCI (National coordination structure for infectious diseases).
- An environmental investigation, in which water samples and environmental swabs were collected from the water supply of the water company, the water supply system of the building where the Flora was held and all water-using products which had been on display at the exhibition and could be traced and adequately cultured.
- A case-control study in which confirmed and probable cases from the case-register were interviewed concerning their visiting behavior at the exhibition and compared to a control group of inhabitants from Stede Broec who had visited the exhibition.
- A "cohort" study, in which persons without LD who had been working on the West-Frisian Flora as volunteer, employee to the exhibition-organization or exhibitor were asked to fill in a questionnaire on their health status before and after the exhibition and their whereabouts during the exhibition. They were also asked to have a paired blood-sample taken to be tested for antibodies against *L. pneumophila* and to send a urine sample for detection of urinary Lp1 antigen.

This report gives the results from the cohort study. The other parts are reported elsewhere[5;6]. The cohort study was primarily designed to find further evidence on the source of the epidemic, supplementing the evidence from the other investigations.

1.1 Elaboration of research questions

The primary research question is: What is the source of the epidemic?
To answer this question we looked at whether there is:

1. a relation between antibody titers of subjects and specific places they have worked, or other “risk” behavior like drinking tab water.
2. a relation between health complaints of subjects and specific places they have worked, or other “risk” behavior.

Furthermore, we will in this report describe the attack rates in exhibitors of:
1. Legionnaire’s Disease (LD),
2. Serological cases of infection with *Legionella pneumophila* in those without LD
3. Health complaints possibly related to Legionella infection, without established LD

Lastly, we also investigated whether there was any evidence of Pontiac fever occurring in this group.

2. Methods

2.1 The West-Frisian Flora and potential sources of *L. pneumophila* transmission

The West-Frisian Flora was held at the auction building (CNB) in Bovenkarspel (community of Stede Broec, province of North-Holland). In 1999 the exhibition was held from Friday the 19th until Sunday the 28th of February and had around 80,000 visitors. The eleven halls of the CNB auction building were supplied with water by two separate systems. The exhibition was held in halls 2, 3, 4, 5, 8, 9 and 13 (see figure 1). In hall 3+4 (consumer fair) and 8 (agricultural fair), a semi-permanent and a temporary polyethylene water supply had been used during the exhibition. In hall 3 and 4, the temperature during the exhibition had been 20 ºC or more, in hall 8 it had been 18 ºC or less, as estimated by exhibitors. During the three months prior to the exhibition, in the eastern part of hall 3 flower bulbs had been kept at 30º C. In hall 5 and 13 (flower show), eleven decorative fountains and a water curtain were spraying. The temperature in this hall was 15 ºC or less, to preserve the flowers.

Figure 1: The layout of the auction building where the West-Frisian Flora was held, indicating the watersupply system and all sampling points of the watersupply in the environmental investigation.

A simple risk assessment was carried out[6] of all products capable of spreading *Legionella spp*, based on interviews of exhibitors on the use of products, using an 8-point scale. For each of the following items one point was given: 1. use of water, 2. use of water at 20-43ºC (the temperature range within which Legionella can amplify to dangerous concentrations), 3. use of water at 37ºC (the optimal temperature for Legionella growth), 4. no disinfection of water at 20-43ºC, 5. no change of water at 20-43ºC, 6. aerosolisation of water at <60ºC, 7. prolonged period of aerosolation of water at <60 ºC, 8. substantial surface for aerosolation of water at <60 ºC. It showed that a whirlpool in hall 3 carried most risk (8 points), followed by a whirlpool in hall 4 (6 points), two baths with bubble-mats in hall 3 and 4 (4 points), eleven fountains in hall 5 and 13 (4 points) and a sprinkling installation in hall 8 (3 points). These products will be considered as potential sources in this report.

2.2 The epidemic of LD

In the outbreak of LD connected to the 1999 West-Frisian Flora, 185 cases (132 confirmed, 53 probable) were reported from all over the Netherlands[5]. Confirmed cases were those with confirmed pneumonia with an atypical infiltrate on chest X-ray and diagnostic evidence of LD (by isolation of *L. pneumophila* from respiratory secretions, detection of Lp1 antigens in urine, or a fourfold rise in Lp1 antibody titers in paired acute-phase serum samples). Probable cases where those with confirmed pneumonia with an atypical infiltrate on the X-ray, but without corroborating laboratory evidence of *L. pneumophila*. Moreover, 58 cases were reported as possible or other LD cases. In those cases symptoms were present, but they did not meet the criteria for probable or confirmed cases, e.g. because laboratory evidence of LD was absent or negative, or because they did not have X-ray established pneumonia. All but one confirmed /probable case had visited the exhibition at or after the 23rd of February (see figure 2). The single exception was a person with a history of COPD and pneumonia's, who had visited the exhibition at the 21st of February. Moreover, the attack rate of LD increased steadily from February 23rd to 27th, although it did fall back somewhat at the 28th. A possible explanation for the latter is that on the 28th (a Sunday) more families with children visited the fair, and therefore visitors on this day were younger and less susceptible. Therefore, it is highly likely that the exposure to *L. pneumophila* increased during the time of the exhibition, reaching the infective dose for a healthy person only after February 22nd. Therefore all analyses in this report were done both looking at all participants, and only at those who worked at the fair after February 22nd. As results were rather similar (as only a minority did only work at the fair before February 23rd), we present only the data based on those who worked on the fair after February 22nd .

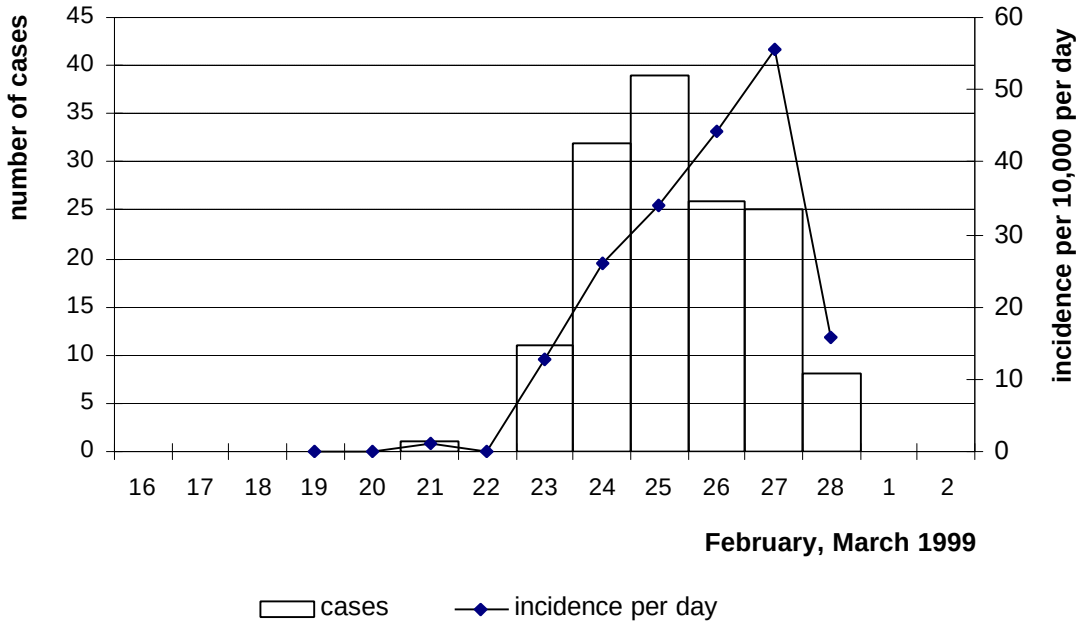

Figure 2: Number of confirmed/probable cases and incidence of LD by day of visiting the West-Frisian Flora

2.3 Study population

2.3.1 Sampling frame and data collection

All firms who were registered as exhibitors on the consumer-products and agricultural fair were contacted by phone and asked to supply the names and addresses of all persons who worked on their stands. Most, but not all firms were reached and responded as requested, although often they supplied work addresses rather than home addresses.
On the 29th of March a questionnaire, a tube for blood and a small jar to sample urine was sent to the 1426 addresses thus collected. The subjects were asked to let their physician take a venous blood sample. They were furthermore asked to fill the jar with urine on March 30th or 31st and send this back. By mistake, EDTA tubes were sent out in this mailing in stead of the intended coagulation tubes. Although the letter for the person taking the blood sample stated that serum had to be sent in, the tubes suggested plasma. Despite the mistake with the tubes, in almost half the cases serum was correctly returned thanks to the alertness of those taking the sample. Of the two types of antibody test performed, only one (the ELISA) can be used both with plasma and serum. The other, the microagglutination test in microtiter, can only be performed with serum, and thus in this first round almost half of the test results on the agglutination test are missing.

On the 7th of April a second mailing and on the 15th of April a third mailing was sent to 82 and 156 persons respectively of firms who had responded later and to persons who were reported by other participants as having worked on the exhibition, or who reported themselves as such. On May 12th, a request for a second blood sample was sent to those who had sent back a completed questionnaire.
The Medical Ethics Committee TNO approved the study in a special procedure for urgent research.

2.3.2 Response

Figure 3 shows the participation in the cohort study. In total 1664 packets were sent to persons who – according to our information - had been present professionally at the West-Frisian Flora, but who were – again to our knowledge at that moment - without confirmed or probable Legionnaire's Disease (LD). Subjects known to have LD were excluded because they had already been approached to take part in the case-control study. However, 3 known cases with LD that had not been approached for participation in the case-control study were sent a questionnaire, but were asked no blood sample as their LD did not need any serological confirmation, while 4 other subjects with LD were unwittingly approached. As LD cases were not systematically approached, we restrict the cohort study to those without LD, thus excluding all 7 persons who were registered as possible, probable or confirmed LD cases in the case register. We will separately describe the LD cases in the cohort in section 6.1.
Of the packages sent out, 29 were furthermore returned as undeliverable and 12 persons received a double package. A total of 905 persons (56% of 1616) participated by either filling in the questionnaire or having a blood sample taken, or both. Furthermore, 180 persons sent back the non-response form, and of 3 others it was reported that they most likely had not worked on the Flora. Analysis of the non-response forms (appendix 2) showed that a fifth of the non-responders had not been present on the Flora at all, and that a considerable part of non-respondents saw the study mainly as a check-up for the presence of Legionnaire's Disease, which they deemed unnecessary because they were convinced not to have this disease. Another reason given for non-response was that the package had arrived too late to have blood taken at the prescribed dates (presumable the dates prescribed for the urine sampling).

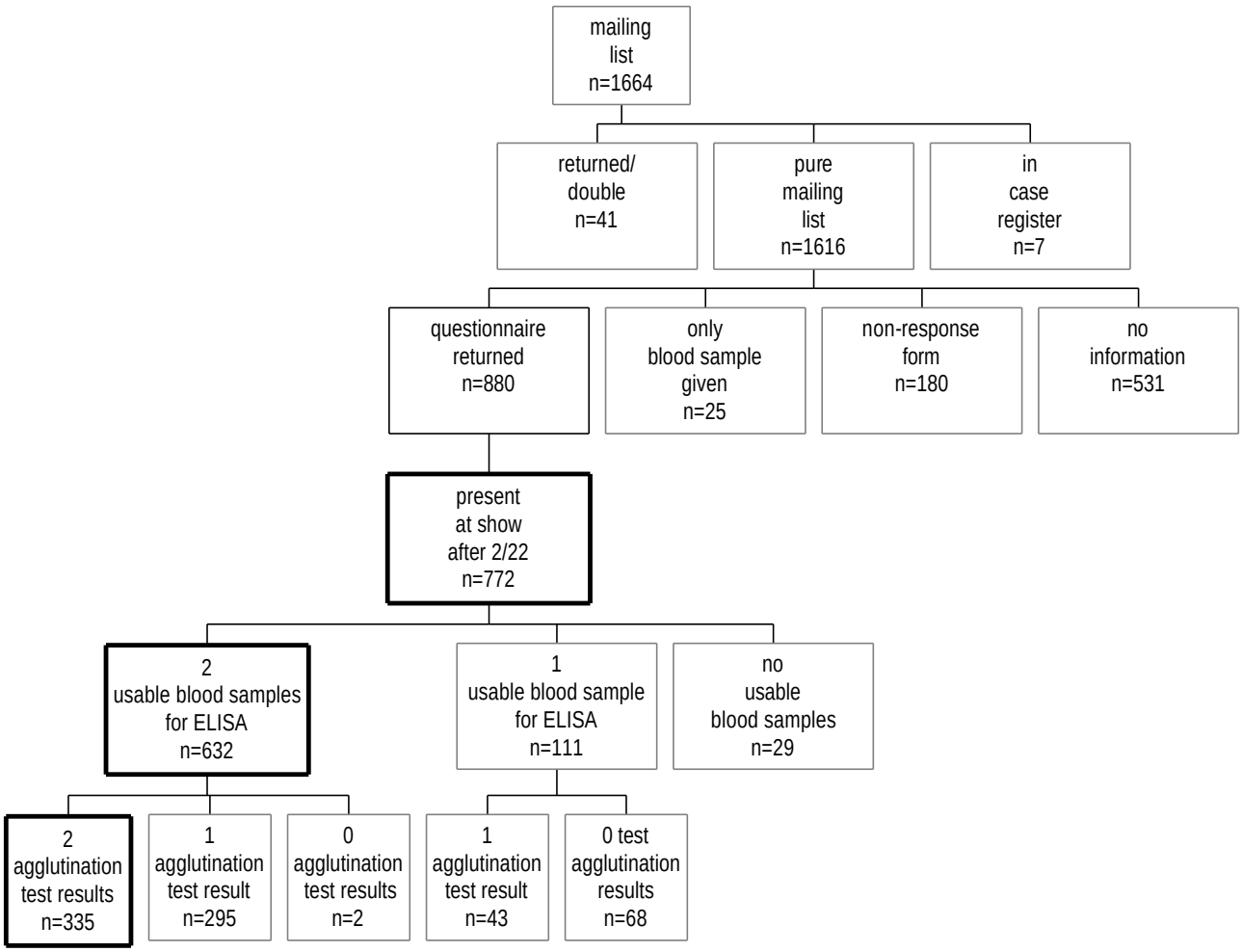

Figure 3: Participation in the cohort study

Of the 905 participants, 25 only gave blood samples, but did not return the questionnaire, and 31 returned the questionnaire but did not give any blood sample. From 849 participants both one or two blood samples and a questionnaire were available. Of the 1574 samples received, 13 could not be analyzed for various reasons. In 5 cases out of those 13 samples, the sample was the only sample present from the person involved, and thus only questionnaire information is available for these 5 persons also. Therefore questionnaire and (one or two) antibody test results were available for 844 persons, of whom 743 (632+111, see figure 3) had been present at the Flora after February 22nd.

Due to the error with the type of tube sent in the first mailing, in the first round plasma but no serum was collected for almost half of the participants. For those participants, only ELISA tests and no micro-agglutination tests could be carried out in the first sample. (See figure 3 for the exact numbers involved). The four main groups used in the analyses (three of them indicated with a stronger border in figure 3) are: Those present after February 22nd with questionnaire data available (n=772); those with both questionnaire data and a usable paired

blood sample (either plasma or serum) available (n=632); those with both questionnaire data and at least one usable blood sample available (n=772) and those with complete information (questionnaire data and two usable samples of paired sera (not plasma), n=335).

2.4 Laboratory methods

The serological investigation comprised of :

1. Titers of *L. pneumophila* specific (serogroup 1 to 7, polyvalent) IgM- and IgG-antibodies as determined with a SERION classic ELISA assays (manufacturer: Institute VIRION-SERION, Würzburg, Germany). This assay yields a quantitative result taking the values <5, >500 or any integer value between 5 and 500. According to the manufacturer, the interserial coefficient of variation is maximally 16%, while the intraserial coefficient of variation is maximally 10%[2]. The following cut-off points are given by the manufacturer[1]:
    - IgM >140: positive; IgM 120-140: borderline.
    - IgG >70: positive; IgG 50-70: borderline.

    However, we are aware of only one paper [10] that evaluates these cut-off points on a rather limited number of subjects by comparing the results to results of an immunofluorescence assay (IFA). From this evaluation the ELISA assay seems somewhat more sensitive than IFA, but the cut-off values mentioned above, although not performing badly, can not be derived from this evidence.
2. Agglutination in microtiter for IgM-antibodies (monovalent) against *L. pneumophila* serogroup 1 (Lp1) and serogrouptype 6 (Lp6). The reason for testing for these two serogroups (SG) was that these were the two serogroups found in the environmental samples. Moreover, in patients only serogroup 1 was found. Cut-off point criteria for micro-agglutination tests are laboratory dependent. A cut-off value stated in the literature (99% specificity) is 1:16 for SG 1 and 1:128 for SG 6 [16]. However, the lowest value in the tests used here is < 1:32, and thus a cut-off value of 1:16 clearly relates to a test performed differently.

The analysis was carried out by the Regional Laboratory for Public Health in Tilburg, under supervision of dr. M.F. Peeters. Paired blood samples from each person were analyzed together.

For epidemiological investigations it is recommended to compare antibody levels to those of a reference population. Therefore 481 bloodsamples were analyzed randomly choosen from a bank of 9948 sera that was established in a cross-sectional population-based nationwide sero-surveillance study carried out in the Netherlands in 1995/1996 (The PIENTER study). The design of this serosurveillance study is described elsewhere in detail[17]. In short, in each of five geographic regions, with approximately equal numbers of inhabitants, eight municipalities were sampled proportionally to their size. Within each municipality, an age-stratified sample (0, 1-4, 5-9,...,75-79 years) of 380 persons was drawn. Eligible individuals

were asked to fill out a questionnaire and to give a blood sample at a special clinic. Blood samples were stored in a refrigerator during the day and at night. The sera were harvested the next day and divided into portions which were stored at minus 86 °C. From this serumbank we took a sample of 480 persons. Thereto 12 samples were choosen from each municipality in a way to assure an age distribution similar to that of the exhibitors. Thus we randomly chose in each municipality one sample from those aged 15-24 years, 10 from those aged 25-64 years, and one from those aged 65-79 years. By mistake, one extra sample was extracted, and in 3 cases a sample was extracted that was not the intended sample. This resulted in 481 samples, which were analysed by the same laboratory that did the analyses of the exhibitor's samples, with the same serological tests, using the same lot number of the commercial ELISA testkit.
The urine samples were tested with Binax now®[20], an immunochromatografic membrane essay, on the presence of antigen for Legionella pneumophila serogroup 1. The analysis was carried out by the Regional Laboratory for Public Health in Haarlem, under supervision of drs. E.P.F. Yzerman. Results of these analyses will be published elsewhere. However, all urine samples from exhibitors without LD tested negative, confirming that the persons studied here were free of LD at that moment.

2.5 Outcome assessment

2.5.1 Serological status

Traditionally, a positive serological diagnosis of infection with *L. pneumophila* is made when there is a four-fold increase in antibody-titers between a sample taken during the first days of the illness and a sample taken several weeks later. In this study two blood samples were taken in order to observe such a seroconversion. However, the first blood samples were taken approximately 1 month after the end of the Flora. Seroconversion of IgG has been described to take place in 58% of converters within 3 weeks after the first symptoms (approximately a month after exposure)[14]. Seroconversion of IgM takes place faster than seroconversion of IgG, with all 35 patients in one study reaching the highest titer within 5 weeks[14]. Therefore it is likely that a large proportion of seroconversion, especially in IgM, will already have taken place before our first sampling time.
On the other hand, up to 14 weeks may be required for seroconversion of IgG[13], and some of those late conversions will fall within the time window of our sampling. However, in some patients with established LD no IgG response is seen at all[22].

Focusing on seroconversion only, we expected that a substantial part of the cases will be missed. The absolute height of the titer is therefore also included as an indicator of recent infection with *L. pneumophila.* Therefore we consider both the dynamics (changes in titers), and the absolute values of the titers.

In an epidemiological study there is no real need to define cut-off values, neither for absolute values, nor for the increase in titer required. Classification of persons into “positive” or “negative”, based on disputable cut-off points only means loss of information and thus reduces the power of analyses. Nevertheless, for a first visual inspection of data and for presentation such a classification is still useful, and therefore such a classification is employed here also.

The cut-off points are defined in terms of percentiles and thus can be used both for the ELISA and the micro-agglutination tests.

2.5.1.1 Cut-off points for absolute values

We made two categories based on absolute values:
- Using as cut-off value the 99th -percentile of the reference population. We will call these “probably serologically positive”.
- Those with values between the 95th percentile and the 99th percentile of the reference population: these are seen as “possibly serologically positive”.

In this definition age-dependent percentiles were used, determined by using a simplified estimation model [24]. In short, after logarithmic transformation, the mean, standard deviation and skewness were modeled separately. In our data, only the mean showed age dependency and no significant skewness was present. Therefore the final model of the antibody distribution consisted of an age-dependent geometric mean (modeled by fractional polynomials) and an age independent standard deviation (on the logarithmic scale). In paragraph 4.5 the values of the percentiles used are given.

For the micro-agglutination tests only a limited number of positive results (= larger than the lowest value) were observed in the reference sample. Thus data were limited and did not allow the determination of age-dependent percentiles. Therefore overall percentiles were used. For both tests (serogroup 1 and 6) the 99th percentile was equal to or less than four times the lowest possible test result. Therefore a fourfold increase or decrease in micro-agglutination titer always includes exceeding the cut-off points for the absolute value.

2.5.1.2 Definition of absolute values

As we have paired titers for most (but not all) participants, it is not self evident which of the two titers to use in the analyses using the absolute height of the titers. When complete time-histories of antibody responses would be available, the best outcome measure would be the peak value of the response. It is clear from the literature that the moment on which this peak occurs varies considerable between subjects, and also differs for IgG and IgM. So while for one person the first titer might be closest to the peak, it may be the second titer for another

person. We therefore decided that the best measure would be the maximum value of both measurements.
However, when we want to combine data from those with only one sample tested, with data from those with two samples tested, this could – in theory – lead to bias, as the maximum of one measurement is expected to be lower than the maximum of two measurements. When we analyze the data from all persons returning blood (including also those of whom only one test result is available), we therefore use the titer of the first or only blood sample.

2.5.1.3 Cut-off points for seroconversion

The traditional criterion for seroconversion is a four-fold rise to an ultimate level of at least one twofold dilution step below the cut-off titer [16]. This criterion is developed for tests using twofold dilution steps, where a twofold rise could easily be due to measurement error. The micro-agglutination test also uses twofold dilutions, and thus it is reasonable to apply the fourfold increase criterion to this test. The ELISA tests, however, are likely to have better discriminatory power, as the coefficient of variation given for intraserial measurements is maximally 10%[2]. However, normal physiological fluctuations need to be considered also in setting such a criterion, but data on such normal fluctuations are lacking. To be safe, we therefore apply the – possibly too conservative – criterion of a fourfold rise to at least the 75th percentile of the reference population. As our measurement period is likely to fall after the peak of the antibody response for many respondents, we also included a fourfold decrease (from at least the 75th percentile) in our definition of seroconvertors. As these criteria might be too strict, we also distinguish a group of "possible" seroconvertors, defined as those with a 2 to 4-fold increase or decrease, also with the highest value above the 75th percentile of the reference population.

2.5.1.4 Case-definition

An overall case definition was made by combining those who fulfilled one of the criteria given above for being probably serologically positive and/or a probable seroconvertor into probable serological cases. Similarly, those who were possibly serologically positive and/or a possible seroconvertor (but not already a probable serological case) are possible serological cases.

2.5.1.5 Assessing probability of infection

In order to study health complaints in those likely to have been infected, we also estimated for each person the probability of having been infected, taking both the antibody levels and the place of work into account. For this we used a Bayesean method. This is described in more detail in appendix 3.

2.5.2 Assessment of health complaints

Detailed information about clinical symptoms, hospital admission, absence due to illness and possible risk factors (underlying disorders, medication and smoking and alcohol use) were collected by questionnaire. Respondents were asked to fill in the date of onset of a symptom and the duration of the symptoms. For analyses we excluded reported symptoms which already started before the 23rd of February or started 20 days or more after the last visit to the Flora.

2.6 Exposure assessment

Exposure mostly relates to the places on the Flora where the subjects have been and the time spent there. In order to find a source, the effects of place of work are most important (2.6.1). Some insight in the effects of the amount of time spent at places is also useful, as this might explain why effects are seen in some subjects, but not in others (2.6.2). Apart from this, some specific risk behaviors are studied (2.6.3).

2.6.1 Exposure characterized by place of work

Our first analyses concern place of work at a general level. For these analyses we assigned each person to the hall where he or she worked. Secondly, we looked in more detail to the place within a hall where a person had worked. This is only possible for those halls where occupied stands were located, as only those working on stands have a more or less fixed place of work. Most of the occupied stands were found in hall 3 and 4 (the consumer products fair) and in hall 8 (agricultural fair).

2.6.1.1 Exposure assessment by hall

Those who worked at a particular stand could be easily assigned to the hall where the stand was situated. Some participants, however, did not work at a stand, but nevertheless reported that they had worked in a single hall. They could not be included in the analyses by stand, but they could be included in the analyses by hall and were assigned to the hall they reported.

Based on the stands and halls reported we divided the participants in the following categories:

1. Those having their main work place at the consumer fair (further subdivide according to whether they worked in hall 3 or 4). We excluded those who worked at stand 3.13, the restaurant that was situated next to, but not inside hall 3 and those who worked at the first aid post (3.03), in a room separated from hall 3 by walls. Hall 4 was considered to include the auction room.

2. Those having their main work place at the agricultural fair (hall 8). In this group we included the persons that worked both at the agriculture fair and the flower exhibition, when they worked for a firm with a stand in hall 8. We included also the persons working in hall 9 (vegetable exhibition and a children's farm).
3. Those having their main work place at the flower exhibition (hall 5 and 13).
4. Those having their main work place elsewhere. This group comprised those working in the first aid post, in the restaurant next to the consumer fair, the ticket office, the parking lot or other places outside the auction building or in halls that were not in use by the exhibition.
5. Those who indicated they had worked in many halls, mostly including the consumer fair. This group included persons organizing the Flora, those involved in security, and others who did not have a fixed working place but walked around. A few persons were included who worked at two or more stands in different halls.

2.6.1.2 Exposure according to the stand where a person was working

Information on the stand number where a subject had worked was available both from self-report in the questionnaire, and from the mailing list, which was compiled through the employer. In general, both were in agreement. In a few cases there were discrepancies and we assumed that an error had been made by the respondent and used the stand number from the mailing list. In all of those cases, the name of the employer listed in the questionnaire was in agreement with the mailing-list.

Six persons reported that they had worked on more than one stand:
1. stand 454 (52.5 hr) and stand 305 (3 hr on first day)
2. stand 830 (4.5 hr) and 345 (4.5 hr)
3. stand 466 (6 hr on the first day) and stand 801 (43 hr)
4. stand 454 (18 hr) and 441 (7.5 hr)
5. stand 802 (11.5 hr) and 801 (0.75hr)
6. stand 832 (27 hr), 833 (13 hr) and 835 (13 hr).

Persons 4, 5 and 6 had worked on neighboring stands, and therefore we assign the exposure fully to the stand where most hours were worked. Persons 1 and 2 were included twice in the analyses that were carried out by stand. For instance, person 1 was included once as a person working 3 hours on stand 305, and once as a person working 52.5 hours on stand 454. In the analyses by hall, we assigned persons 1, 2 and 3 to the multiple hall group, person 4 to hall 4 and person 5 and 6 to hall 8.
The effects of these assumptions on the results are negligible, as for person 3 no blood samples were available, and none of the other five persons had antibody titers above the threshold where they would be indicative of recent infection with *L. pneumophila* according to the manufacturers cut-off points.

2.6.2 Exposure assessment by number of hours spent at the consumer fair

All subjects were asked for each day separately how much time they spent on their place of work (stating times of starting and leaving), and also how much time they spent away from their workplace in other halls. From this, we calculated the amount of time spent in hall 3, hall 4 and hall 8 (the three most common workplaces) and in all other halls together. We calculated this both over the entire period and only for the period after February 22nd (starting on February, 23rd). We also calculated the total amount of time spent at any place on the Flora.
In screening the questionnaires, it became obvious that most respondents included the time at their workplace in their answer on the time spent in each hall away from their workplace. In calculating the hours spent in each hall, we therefore first assigned the time indicated for each hall. If the total time spent at the exhibition on a certain day was larger than the sum of the times indicated for each hall, the excess was assigned to the hall where the workplace was situated.
For most respondents the hours spent at the exhibition, and at hall 3 and 4 thus could be calculated. Approx. 5% of respondents indicated their presence on certain mornings, afternoons or evenings, but did not specify any times. Those respondents were assigned the average duration of a stay during a morning, afternoon or evening.

Times within each hall can be calculated based on the time spent during the entire duration of the exhibition, or only based on the time after February 22nd, when exposure is most likely to have been present. In this report, we only present the latter, but conclusions remain the same when time before February, 22nd would have been included.

2.6.3 Exposure to other risk factors

Apart from accounting for the total time spent on the Flora, participants were asked whether they visited specific stands (including all stands where water was used), and how much time they visited these stands. This information was not asked by day. These data are included in a separate multivariate analysis. Furthermore, participants were asked whether they had drunk any water from tabs on the Flora, and whether they had been in contact with potting compost. The answers on these questions were analyzed separately.

2.7 Assessment of confounding variables

Risk factors for Legionnaire's Disease are treated as potentially confounding variables in this study. However, when the outcome used is antibody level rather than health complaints, it is not clear whether this is correct. It is possible that immunosuppressive factors increase the

risk of contracting Legionnaire's disease, while they decrease the magnitude of the immunoresponse. For other factors, as lung diseases and smoking, it is more likely that they decrease the clearance of *Legionella spp* from the lungs and thus might enhance the possibility for the organism to invade the body. Therefore we decided not to use a single variable for underlying disease, but to split this up in two variables: immunosuppressive disease (defined as chronic renal failure, kidney dialysis, HIV infection/AIDS, history of organ transplantation, cancer with immunosuppressive chemotherapy or radiotherapy in the previous year, use of immunosuppressive medication) and chronic lung diseases (defined as chronic obstructive pulmonary disease, history of pneumonia, sarcoidosis or other severe lung disorders).
Other confounding variables included were age (in the form of a polynomial or spline function of age, in order to prevent residual confounding), gender, current smoking, and alcohol use.

2.8 Statistical analysis

Comparisons between multiple groups were done using chi-square tests (discrete variables) or ANOVA (continuous variables). More specific contrasts between two groups (e.g. hall 3 against hall 4) were tested with the chi-square test or the student t-test, without adjusting for multiple comparisons. However, full p-values are presented in order to allow the reader to apply such an adjustment if he/she so requires.

Adjustment was carried out by multiple linear regression for continuous outcome variables and by logistic regression for binary outcomes.
All titer values were log-transformed before every analysis in order to assure reasonable normality of the data. However, the results are transformed back into the original units (by taking the exponent) for presentation. In all analyses the outcome of >500 U/ml was coded as 500 U/ml, and the outcome of <5 U/ml as 2.5 U/ml.

For visual inspection of the data, the height of the geometric mean titer for each stand was indicated on the map of the consumer fair. As too much noise is present for a clear interpretation of this map, perception of a possible pattern is aided by using a LOESS-type smoothing algorithm[7]. This algorithm finds the N nearest subjects (neighbors) of each grid point and calculates a weighted mean of the titers, with the weight being higher when the particular neighbor is closer to the gridpoint. The tricube function was used as weighing function[7], with the maximum distance used in this function taken as 1 meter more than the distance to the farthest neighbor, in order to prevent major loss of information from tied observations. A simple 0-order LOESS was used, which implies that a weighted mean is used, and not the predicted value from a weighted linear or quadratic regression. Apart from computational ease, this has the advantage that only the distance between points needs to be

used, and no directional information is needed. This enables us to include the placement of the wall separating hall 3 and hall 4 in the algorithm, by calculating the distance from a point in hall 3 to that in hall 4 as the distance of the shortest route through one of the passageways. For the agricultural fair this allows taking the L-shape of the hall into account.

3. Descriptive results on the study population

3.1 Characteristics of the participants

Table 1 shows the characteristics of the participants included in the analyses. There were significant differences in age and gender between the groups working in different halls, with more women working at the consumer fair, and younger persons working at the agricultural fair.

Table 1: Characteristics of the study population by main place of work.

	Main workplace in hall 3 n=151	Main workplace in hall 4 n=249	Main workplace agricultural fair n=246	Main workplace at flower show n=35	Other workplaces n=43	Worked at multiple locations n=48	All n=772	Only those with paired samples n=632
Age group								
% <16	1.4	1.2	1.2	0.0	0.0	2.1	1.2	0.8
% 16-29	14.3	10.6	24.2	2.9	9.3	8.5	15.1	14.0
% 30-39	16.3	18.0	25.0	17.7	18.6	19.2	20.0	19.1
% 40-49	19.7	24.1	26.2	38.2	30.2	25.5	25.0	24.6
% 50-59	31.3	33.1	18.4	14.7	30.2	36.2	27.2	28.6
% 60-69	13.6	11.0	4.5	20.6	11.6	8.5	9.7	11.2
% 70+	3.4	2.0	0.4	5.9	0.0	0.0	1.7	1.8
% males	45.3	38.9	70.5	91.4	32.6	79.2	54.8	54.0
% smokers	29.1	37.4	33.3	28.6	34.9	27.1	33.3	32.6
% using alcohol	65.9	69.6	80.1	90.6	54.8	82.6	73.2	72.6
% immunologically compromized*	3.3	4.8	1.2	0.0	2.3	0.0	2.7	2.9
% with lungdisorder	23.2	16.9	12.2	8.6	11.6	12.5	15.7	16.1
% with two blood sample results	77.5	82.7	81.3	74.4	91.3	80.3	81.9	100.0

*: Immunologically compromised was defined as chronic renal failure, kidney dialysis, HIV infection/AIDS, history of organ transplantation, cancer with immunosuppressive chemotherapy or radiotherapy in the previous year, use of immunosuppressive medication

The group who worked at multiple places consisted mainly of men, which is understandable from the composition of this group (fire brigade, security personnel, members of the organizational committee, persons setting up and dismantling the exhibition).
Alcohol use, but not smoking, differed between the groups. The amount of lung disorders (defined as COPD, history of pneumonia, sarcoidosis or another serious lung disorders) also

differed between the halls. This partly reflects the different age structure of the groups working in different halls. However, when taking age en gender into account, a higher prevalence of lung disorders is still observed in hall 3 compared to the other halls. Only a few persons were immunologically compromised, and no statistically significant differences existed between the halls.

3.2 Description of exposure

The majority of time was spent in the hall where the respondents worked (table 2), although several respondents working in hall 8 stated that they had spent a substantial amount of time in hall 5, and several persons working at the flower exhibition (hall 5 and 13) indicated that they had spent substantial time in hall 8. Respondents were also asked whether they had visited a large number of specific stands, and for how much time. The mean amount of time indicated for all stands where water was used is also given in table 2. This question was not completed or only partly completed by many respondents and therefore the times given will underestimate the real amount of time, as respondents who did not fill in a time were assumed not to have spent any time at a stand. The results therefore can only be taken as an indication of visiting behavior of the groups working in different halls. Table 2 shows that respondents reported that they visited stands in their own hall more often than stands in other halls. Those working on the flower exhibition stated that they visited stands in the neighboring halls 4 and 8 more often than stands in hall 3. More detailed examination also showed that when someone spent substantial time at another stand than their own, this other stand often was a neighboring stand in the same hall.

Those working at multiple places visited stands in all halls relatively often, while those working at other places visited stand in all halls only rarely. However, several of the latter indicated that they had their coffee breaks at the Flora office next to hall 3 (3.05).
Those working in several places contained a subgroup which spent many hours (up to 30 hours) at specific stands near the places of entry and exit of each hall (301, 454, 845 and 848).

Table 2: Exposure characteristics of the study population by main place of work: mean duration in hours (first 8 rows) and minutes. Within brackets the standard deviation.

	Main workplace in hall 3 N=151	Main workplace in hall 4 n=249	Main work place at agricultural fair n=246	Main workplace at flower show n=35	Other workplace n=43	Worked at multiple places n=48	All n=772	Those with two samples n=632
Hours on Flora	43.2 (36.1)	37.0 (34.9)	26.5 (23.5)	17.9 (11.6)	34.0 (32.3)	29.9 (33.5)	33.7 (31.9)	33.2 (31.3)
Hours in hall 3	41.0 (35.6)	1.2 (2.5)	0.5 (1.0)	0.8 (0.8)	2.4** (7.3)	6.9 (18.3)	9.8 (23.3)	8.8 (22.1)
Hours in hall 4	0.8 (1.1)	33.8 (33.1)	0.6 (1.6)	1.0 (1.3)	0.8 (1.5)	2.6 (7.5)	11.8 (24.7)	11.3 (23.8)
Hours in hall 8	0.3 (0.7)	0.4 (0.7)	22.4 (23.0)	2.1 (2.0)	0.4 (0.9)	4.9 (14.5)	7.4 (16.6)	7.8 (17.2)
Hours at Flora after 22-2-1999	32.3 (21.8)	27.0 (22.5)	21.2 (16.3)	11.6 (7.6)	23.0 (20.1)	21.0 (20.0)	24.9 (20.4)	21.6 (20.4)
Hours in hall 3 after 22-2-1999	30.5 (21.7)	0.8(1.2)	0.4 (0.6)	0.7 (0.6)	1.6 (4.5)	4.7 (11.9)	6.7 (15.5)	5.5 (14.0)
Hours in hall 4 after 22-2-1999	0.7 (1.4)	24.8(21.7)	0.4 (1.2)	0.7 (0.6)	0.6 (1.0)	1.8 (4.4)	8.4 (16.8)	7.1 (15.4)
Hours in hall 8 after 22-2-1999	0.2 (0.5)	0.3(0.5)	18.1 (16.2)	2.0 (1.9)	0.3 (0.5)	4.0 (9.4)	6.3 (12.5)	5.7 (12.3)
Minutes visiting stand 309	3.6	1.3	0.4	0.3	0.9	3.3	1.5	0.9
Minutes visiting stand 405	0.7	4.4	0.9	2.0	0.7	4.1	2.2	2.4
Minutes visiting stand 848	0.2	0.3	1.6	3.5	0.8	92.1*	6.6	7.8
Minutes visiting stand 327	4.4	0.7	0.3	0.4	0.5	1.9	1.3	1.1
Minutes visiting stand 335	2.6	1.0	0.4	0.4	1.0	3.3	1.2	1.3
Minutes visiting stand 337	3.1	0.8	0.4	0.3	0.5	2.2	1.2	1.0
Minutes visiting stand 355	1.6	0.8	0.4	0.1	0.9	2.2	0.9	1.0
Minutes visiting stand 411	1.1	3.5	0.6	1.4	0.8	3.0	1.8	1.9
Minutes visiting stand 414	0.8	3.5	0.6	1.1	0.2	2.6	1.7	1.8
Minutes visiting stand 448	0.5	0.6	0.2	0.3	0.3	1.9	0.5	0.4

* due to 5 persons from security who spent many hours near this stand, which was situated next to an exit of the exhibition.

** due to 3 persons from the first aid post who stated to have spent almost half or more of their working hours in hall 3

4. Results of the serological study

We will first describe the antibody levels as such. Differences between groups in exposure to *L. pneumophila* will result in differences in the level of antibodies present. No judgment on whether a particular rise in antibody level in a particular individual represents evidence or not of infection with *L pneumophila* is needed to make these comparisons.
Levels of antibodies will be first compared between broadly defined groups (between persons with their main workplace in different halls, (paragraph 4.1) and by the time worked in the three main halls (3,4 and 8) (paragraph 4.2). In paragraph 4.3 we will look more in detail to the relation between the place within those halls and antibody level, while in paragraph 4.4 we will look at the effect of other possible exposures (as drinking water) on antibody levels.

Secondly, we will use the case definition given in 2.5.1.4 to indicate probable and possible serological cases. In order to do so, we first need to define the percentile values of the reference population (4.5). In paragraph 4.6 we will then look at the distribution of the probable and possible serological cases over the halls, and within hall 3 and 4.

4.1 Antibody levels by hall

Table 3 shows the results of the serological tests by main workplace for the ELISA tests, table 4 the results for the agglutination tests. ANOVA showed that statistically significant overall differences in ELISA results existed between workplaces (p-value <=0.0001 for all ANOVA's).
In table 4 the percentage of respondents with a micro-agglutination test result above the 99th percentile of the serumbank samples are given. Fisher's exact test shows that there are statistically significant overall differences between halls (p=0.005) for the micro-agglutination results for serogroup 1, but not for serogroup 6. As all serotyped isolates of patients were serogroup 1, we will not further analyze the serogroup 6 results.

Table 3: Absolute values of geometric mean ELISA antibody titers against L. pneumophila.

Group	IgG-Elisa in U/ml		IgM-Elisa in U/ml	
	In first or only sample (n=772)	Maximum of paired samples (n=632)	In first or only sample (n=772)	Maximum of paired samples (n=632)
Hall 3	30.6	36.4	25.8	27.3
Hall 4	18.6	20.2	14.2	14.6
Agricultural fair	20.2	23.0	15.2	16.3
Flower show	17.3	20.8	15.2	17.5
Other places	15.9	19.4	14.1	15.4
Several halls	19.3	21.0	17.7	17.4
All	20.8	23.5	16.6	17.4
Reference population (n=481)	15.1		11.5	

Table 4: Percentage (number) of exhibitors with a agglutination antibody titer against L. pneumophila above the 99th percentile of the reference population in the first or only blood sample analyzed.

Group	IgM Lp1 1: 32 or more	IgM Lp6 1:64 or more
	(n=673)	(n=673)
Hall 3	7.1 (9)	2.0 (2)
Hall 4	0.5 (1)	1.2 (2)
Agricultural fair	1.4 (3)	1.8 (4)
Flower show	0.0 (0)	0.0 (0)
Other places	0.0 (0)	2.4 (1)
Several halls	2.3 (1)	2.3 (1)
All	2.1 (14)	1.5 (10)

For comparison, table 3 also contains the results for the serumbank sample, which yielded a geometric mean titer for ELISA IgG of 15.1 U/ml and a geometric mean ELISA IgM titer of 11.5 U/ml. This means that in all halls the geometric mean titer is increased compared to the titer in the reference population. However, due to the sometimes small number of persons in some of the halls, the difference is not statistically significant for all groups. The titers in hall 3 are clearly higher than those in the other workplaces ($p<=0.0001$). When hall 3 is omitted from the population, no statistically significant differences in titers between halls remain, but the titers of those not working in hall 3 are statistically significantly higher than those observed in the serumbank sample. The titers of respondents in hall 4 are not higher, and even

slightly lower than those of respondents working in other places (excluding hall 3): the difference, however, is not statistically significant.

When those who were not present at the Flora after February 22nd are included (see table 29 in appendix 4), the mean titers decrease in those from hall 3, while changes are minimal in the other groups (with the possible exception of those who had worked in several halls). This confirms the belief that exposure was highest after February 22nd.

A regression model is used to adjust the results for the potential confounders age, gender, smoking, alcohol use, lung disorders and immunosuppressive disease/medication. Models containing all these variables again indicate only statistically significantly increased titers in those working in hall 3. Table 5 gives the ratio of the antibody level of those working in hall 3 compared to those working in all other places, as calculated from a regression model containing all potentially confounding variables. However, it should be noted that the residuals for the IgG models showed some deviations from normality. As regression analysis is rather robust to violations of the assumption of normality, this is not considered to make the results unreliable.

Table 5: Ratio of titers in those in hall 3 compared to other groups adjusted for age, gender, immunosuppressive disease/medication, lung disorders, smoking and alcohol use.

	ratio hall 3 /all other places	
	First sample	Maximum paired sample
IgG-Elisa	1.58 [1.31-1.91]	1.67 [1.36-2.04]
IgM-Elisa	1.81 [1.54-2.13]	1.88 [1.56-2.26]

In table 6 the mean changes in antibody titers between the two measurements are given. In the column labeled “increase” the mean percentage of increase (which is negative in case of a decrease) from the first to the second measurement is given. In calculating the mean increase, a 100% increase (doubling) is cancelled out by a 50% decrease (halving) in another person. Thus the numbers in the column increase indicate whether there was an overall increase or decrease in antibody titer in the group. In the column “change” we present the average change, where the direction of the change is not taken into account. Here a 100% increase is the same as a 50% decrease. In other words, “change” is a measure for the overall dynamics of the titer heights within the group.
Similarly, in table 7 the number of 4-fold increases and 4-fold changes (= number of 4-fold increases and 4-fold decreases) in agglutination titers against Lp1 is given. It shows a (statistically non-significant) higher number of cases of 4-fold decreases in hall 3 compared to other halls.

Table 6: Changes in antibody titers against L. pneumophila between 4 and 10 weeks after the end of the Flora.

Group	IgG-ELISA		IgM-ELISA	
	Increase	Change	Increase	change
Hall 3	3.4	31.0	-10.5*	34.6
Hall 4	-3.5	18.9	- 4.6*	18.1
Agricultural fair	4.2	18.7	-2.3	22.6
Flower show	6.4	28.1	-7.1	32.4
Other places	15.6*	24.7	7.1	16.8
Several halls	-2.9	13.4	-5.1	13.6
All	1.8	21.4	-4.4**	22.7

* = p < =0.05 when testing against increase=0 (only applicable for increases).
** = p <=0.005 when testing against increase=0 (only applicable for increases).
*Note: Increase is defined as (exp(mean(log(T2/T1)))-1)*100% and change as (exp(mean(abs(log(T2/T1))))-1)*100%, were T1 is the titer at 4 weeks, and T2 is the titer at 10 weeks (see text).*

Table 7: Percentage (number) of 4-fold increases and changes in agglutination IgM antibody titers against L. pneumophila serogroup 1 between 4 and 10 weeks after the end of the Flora.

Group	IgM Lp1 prevalence of 4-fold increase (n)	IgM Lp1 prevalence of 4-fold changes (n)
Hall 3	0 (0)	5.9(4)
Hall 4	0 (0)	0 (0)
Agricultural fair	1.0 (1)	1.9 (2)
Flower show	0 (0)	0 (0)
Other places	0 (0)	0 (0)
Several halls	0 (0)	0 (0)
All	0 (0)	0 (0)

Note: Change is defined as a 4-fold increase or a 4-fold decrease.

Overall, there is a slight decrease in IgM titers and a slight increase in IgG titers during the 6 week period. However, only the decrease in IgM is statistically significant.
Although the overall increase in IgG antibodies is not statistically significant, the table shows that this stability is due to the titers increasing in some halls, while decreasing in others. ANOVA shows that these differences between halls in increases of IgG are statistically significantly (p=0.04). The magnitude of increases in IgG in hall 3 takes a middle position between all halls, and therefore it is not surprising that contrasting the increase in IgG in hall 3 with that in all other halls does not yield a statistically significant difference.
ANOVA shows no statistically significant differences between the decreases in IgM between halls. And although the decrease in largest in hall 3, the difference with other halls is not statistically significant.

More effects are seen when we look at the magnitudes of the changes (irrespective of direction): the change in both IgM and IgG antibodies differs statistically significantly between halls. In both cases, changes are largest in hall 3. P-values for comparing hall 3 with all other halls are p=0.02 (IgG) and p=0.009 (IgM). When the last analysis is done multivariately, adjusting for the potential confounders, the p-values are even lower (p=0.002 for IgG and p=0.0008 for IgM).

4.2 Antibody levels by duration of exposure

Table 8 shows the results of the serological tests by time spent in the three main halls, for the respondents that did not work in that particular hall. Thus, in calculating the titers by time spent in hall 3, those working in hall 3 and those working at multiple places are excluded. Table 9 shows the complement, the results of the serological tests by time spent in the three main halls, for only the respondents that worked in that particular hall.
Both tables show only very weak, not statistically significant relations between antibody titers and duration of exposure. In both tables, there seems to be a lower titer in those spending less than 2 hours in hall 3 than in those spending more time in hall 3. When all those spending less than 2 hours are compared with all those spending more than 2 hours in hall 3, there are significant differences seen in IgM titers among exhibitors from hall 3, and in IgG titers in exhibitors not working in hall 3, while there is a borderline significant effect in IgG titers for those working in hall 3 (p =0.07 and 0.09 respectively for first or only sample, and the maximum of two paired samples). Nevertheless, those working in hall 3 seem to have a higher titer than those working elsewhere, even among those who said to have spent less than 2 hours in hall 3 after February 22nd. The number of hours spent in hall 4 or 8 does not seem to increase the titers.
When combining the two tables into one, not surprisingly a strong relation between antibody titers and duration of exposure appears, as those in hall 3 (with higher titers) fall mostly in the long duration categories, and those not working in hall 3 fall mostly in the lower categories.

Table 8: Serological results by time spent in the three main halls after February, 22nd (those working in the particular hall are excluded).

Group	IgG-Elisa (geometric mean)		IgM-Elisa (geometric mean)	
	First or only sample	Maximum of paired samples	First or only sample	Maximum of paired samples
Time in hall 3	o			
0 (n=128)	20.3	23.0	13.2	13.2
0-0.5 hrs(n=199)	17.8	21.0	15.0	16.5
0.5-1 hrs (n=107)	20.2	21.8	16.8	17.0
1-2 hrs (n=81)	17.0	17.7	13.9	14.2
> 2 hrs (n=32)	28.7	31.8	15.6	17.1
Time in hall 4				
0 (n=111)	23.9	27.7	17.3	16.3
0-0.5 hrs (n=180)	22.0	25.4	17.9	20.3
0.5-1 hrs (n=104)	21.1	26.1	20.2	22.2
1-2 hrs (n=61)	23.0	24.3	20.0	16.4
> 2 hrs (n=18)	21.2	22.6	12.6	18.2
Time in hall 8				
0 (n=218)	21.6	24.6	16.6	16.6
0-0.5 hrs (n=144)	22.2	25.0	18.2	18.8
0.5-1 hrs (n= 54)	17.6	20.4	19.3	21.3
1-2 hrs (n= 34)	23.5	25.2	17.2	18.5
> 2 hrs (n=26)	19.6	21.9	14.3	17.1

o = 0.05 < p-value ANOVA <0.1

Table 9: Serological results by time spent in the three main halls after February, 22nd (only for those working after February 22nd in the particular hall).

Group	IgG-Elisa (geometric mean)		IgM-Elisa (geometric mean)	
	First or only sample	Maximum of paired samples	First or only sample	Maximum of paired samples
Time in hall 3			o	o
<2 hrs (n=6)	14.0	14.8	11.3	9.6
2-10 hrs (n=39)	33.0	38.0	30.6	30.8
>10 hrs (n=106)	31.3	37.6	25.7	27.7
Time in hall 4				
<-2 hrs (n=11)	14.3	20.7	14.5	14.6
2-10 hrs (n=91)	19.0	20.9	13.3	14.0
>10 hrs (n=145)	18.7	19.6	14.7	14.8
Time in hall 8				
<2 hrs (n=13)	16.4	17.3	19.1	23.1
2-10 hrs (n=89)	19.8	23.3	15.4	17.2
>10 hrs (n=137)	21.0	23.2	15.0	15.8

o = 0.05 < p-value ANOVA <0.1

Also multivariately this effect is seen. If the influence of duration of exposure is studied by entering simultaneously in a regression model: the total number of hours spent at the entire Flora after February 22nd, and the number of hours spent in this period in hall 3, 4 and 8 respectively, and all potentially confounding factors, the number of hours spent in hall 3 is statistically significant (p-values ranged from <0.0001 to 0.007) in all 4 models (IgG and IgM, paired sera only or first or only sera). The other times do not even approach statistical significance. When we include a variable indicating whether hall 3 was the main work place, the variable for main workplace was highly statistically significant (p-values from <0.0001 to 0.0008), while that for the number of hours spent in hall 3 after February 22nd was no longer statistically significant. However, when we include the number of hours spent in hall 3 not as a continuous variable, but as a binary variable with two categories (two or more hours and less than two hours), this indicator variable becomes highly significant in the IgG models, while working in hall 3 no longer has any influence. In the IgM models the indicator variable for more than 2 hours exposure is also statistically significant, but not so strongly (p-value 0.03 in both models) while working in hall 3 is of borderline statistical significance (p-values 0.04 and 0.10 respectively). As both variables (working in hall 3 and spending more than 2 hours in hall 3) are highly correlated (ρ=0.85), including them both in the model will decreased the power of estimating the effect of either of them.

We calculated the correlation coefficients between absolute titers and the number of hours in hall 3. We did the same for the dynamic measures (increase and change in antibody levels). For the absolute measures correlation coefficients ranged from 0.14 to 0.18 (all very statistically significant). For the dynamic measures the correlation coefficients ranged from –0.03 to 0.14. Only the correlation coefficients for measures of change were statistically significant. As the associations with change in antibody levels are weaker than those with the absolute level, analysis of changes is not likely to provide more insight than analysis of the absolute levels only. We therefore did not pursuit these analyses.

4.3 Antibody levels by stand

In figure 4 the geometric antibody levels by stand are given for hall 3 and 4. Due to the small numbers of subjects per stand it is difficult to perceive a pattern in these data.
Therefore a smoothing algorithm was developed (see methods section), in which a weighted average is calculated for each grid point of the map for the 35 nearest subjects to this point. Figure 5 gives the results of this smoothing procedure for the consumer-products fair, and figure 6 for the agricultural fair. The latter show only a slight elevation of IgG titers around stand 8.02, but not around stand 8.48 (upper right corner) where the *L. pneumophila*-positive sprinkler system was located.
In contrast, the maps of the consumer hall show several “hot spots”, especially in hall 3.

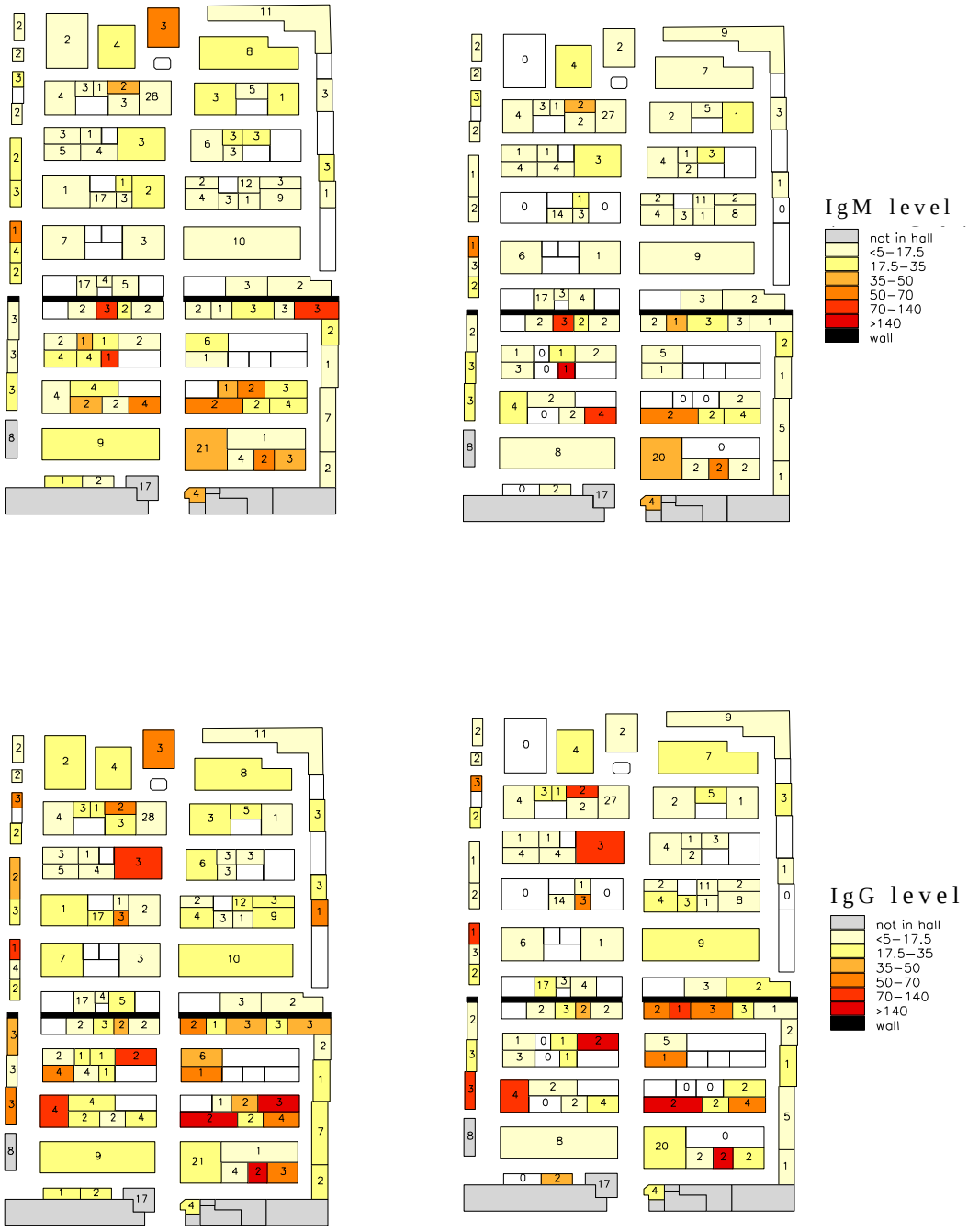

Figure 4: Geometric mean antibody levels by stand. Left: of first or only sample; right: of the maximum value from two paired samples. Upper figures: IgM. Lower figures: IgG.

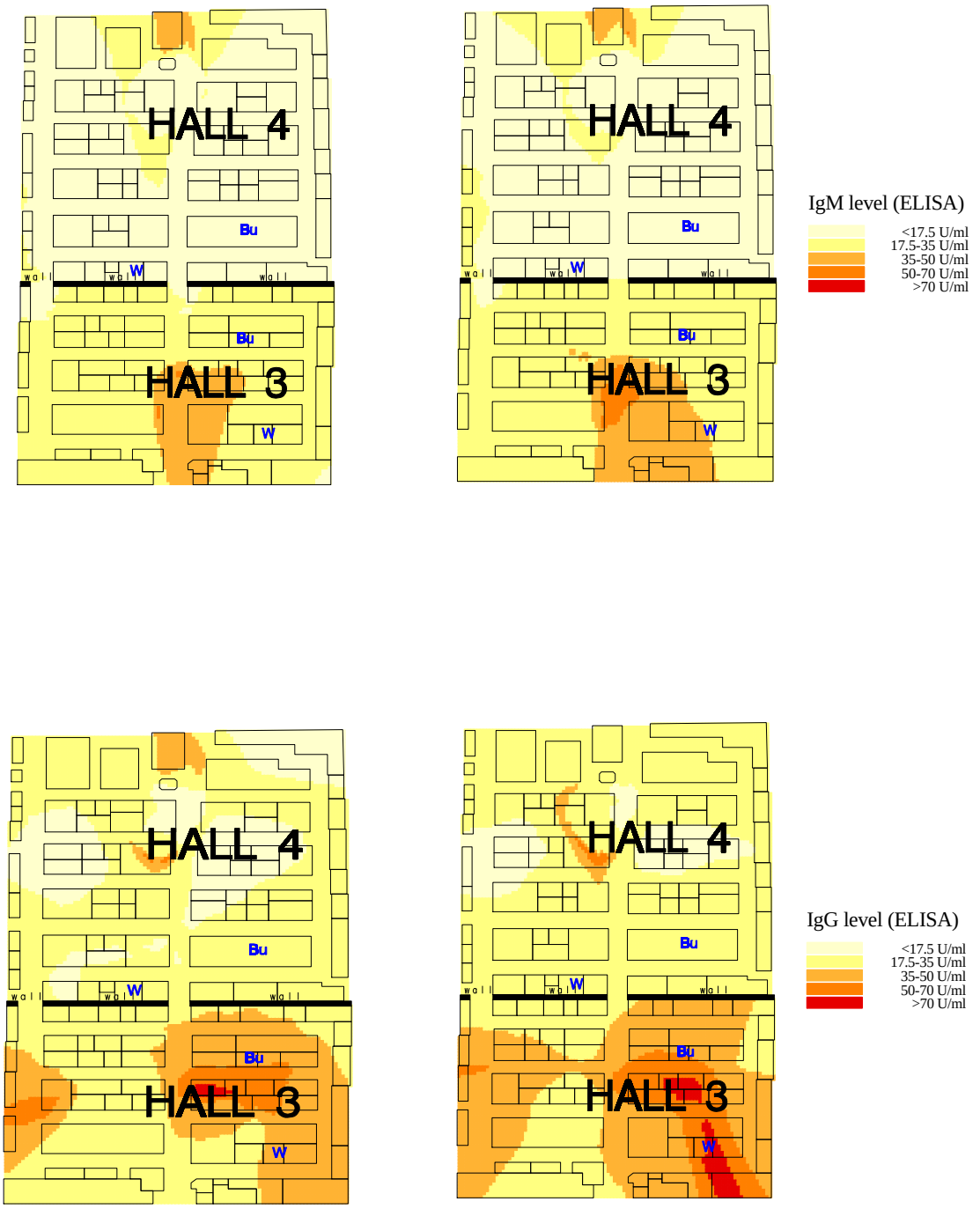

Figure 5: Smoothed antibody levels in exhibitors in hall 3 and 4. W = whirlpool; Bu = Bath with Bubblemat. Left: first or only sample; right: maximum of paired samples. Upper figures: IgM; lower: IgG

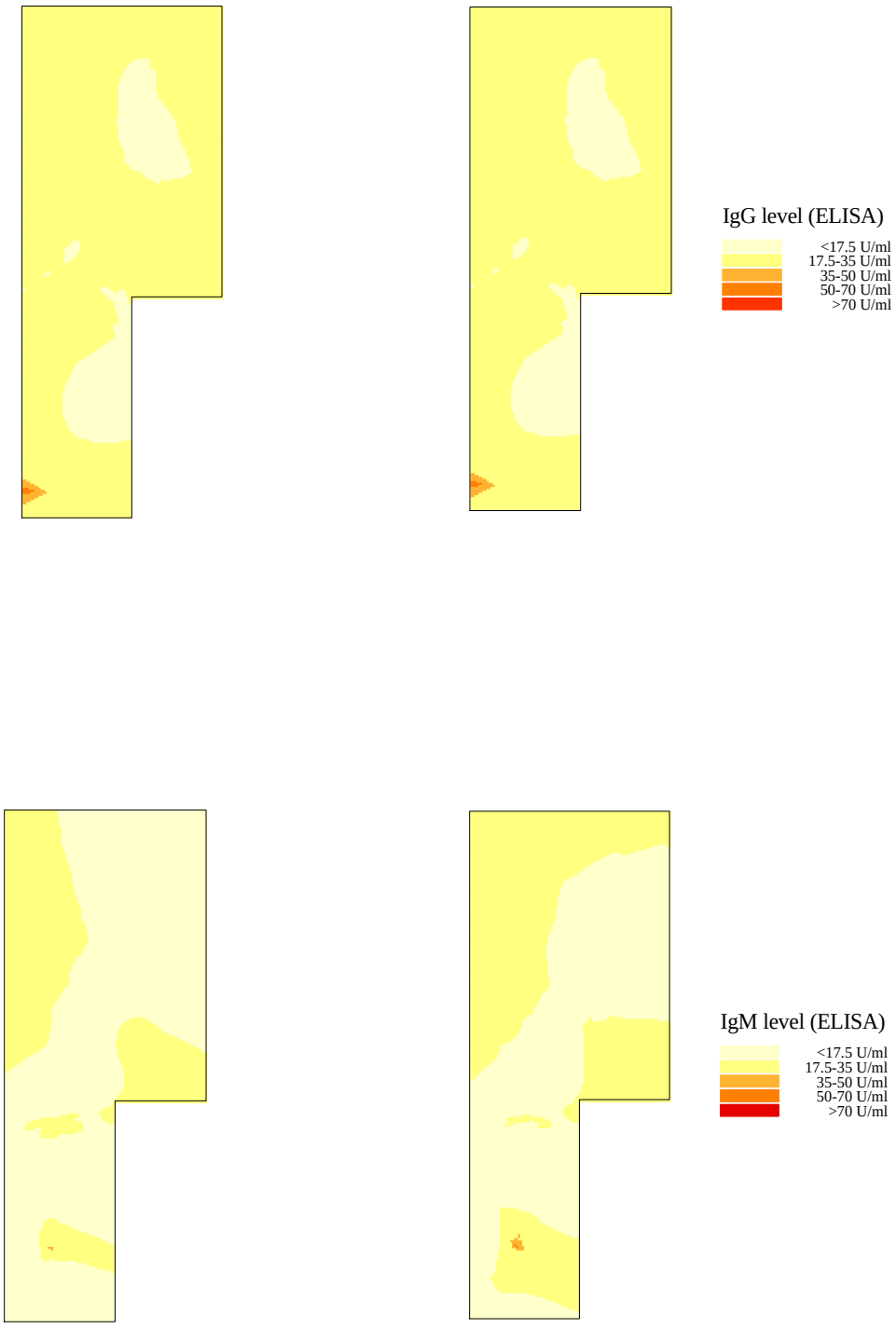

Figure 6: Smoothed geometric mean antibody levels in hall 8. Left: of first or only samples; right: of the maximum of paired samples. Upper figures: IgG; Lower figures: IgM

In a regression model we entered the distance to a stand as a variable. However, only one distance is entered in the model simultaneous, as interpretation of the results becomes difficult when two distances and especially when three distances are entered, because distances form a Euclidean space. Because of this, two distances could represent together the distance to a third stand. Therefore only a single distance is entered in each model.

In table 10 we give the explained variance of the models when entering the specific distances (one at a time), as well as the significance of the distance. The models indicate that the titers are significantly higher when closer to both the bath with the bubble-mat and the whirlpool in hall 3. The distances to both appliances is highly correlated (ρ=0.93). Although the fit of the model with the distance to the whirlpool in hall 3 is slightly better, the difference is not large enough to be conclusive.

When the distance to the whirlpool in hall 3 and the distance to the bath with the bubble-mat in hall 3 are entered in the model simultaneously, the IgM-models yields a statistically significant negative effect for the distance to the whirlpool ($p<0.0002$) (i.e. higher antibodies nearer to the whirlpool), and a (borderline) significant protective effect of the distance to the bath with the bubble-mat. In the IgG-models only the distance to the whirlpool is of (borderline) statistical significance.

Table 10: R^2 (explained variance) and p-value for the distance to a particular stand for the models including each a single distance to one of the four stands with potentially Legionella spreading appliances.

Included distance:	IgG-Elisa				IgM-Elisa (geometric mean)			
Distance to:	First samples		Paired samples		First samples		Paired samples	
	R^2	p-value	R^2	p-value	R^2	p-value	R^2	p-value
Whirlpool hall 3	0.085	<0.0001	0.110	<0.0001	0.138	<0.0001	0.165	<0.0001
Bubble mat hall 3	0.077	0.0003	0.093	<0.0001	0.089	<0.0001	0.129	<0.0001
Whirlpool hall 4 *)	0.044	0.3	0.047	0.5	0.071	0.04	0.050	0.05
Bubble mat hall 4 *)	0.051	0.07	0.055	0.08	0.111	<0.0001	0.131	<0.0001

Adjusted for age, gender, smoking, alcohol use, chronic lung disease and immunosuppressive disorders
*) The coefficients are positive, indicating that titers are lower when closer to this source

Furthermore, the questionnaire also asked after the number of minutes respondents had visited a large number of specific stands. We used these data in a regression model including only exhibitors not working in hall 3 or 4. We included in this model the number of minutes respondents indicated that they had spent at the four stands with potentially *L. pneumophila* spreading devices as a visitor. The participants were not asked for the date that they visited the stands. However, we changed the visiting time to 0 when a person had indicated elsewhere that they did not spent any time in the hall were the stand was located after February 22nd . The model included, apart from the potentially confounding factors and the

minutes visiting the stands, the total number of hours one had spent in hall 3 after February 22nd.
No statistically significant effects of visits to these stands were seen in this model.

4.4 Antibody levels by other exposures

We also looked at other possible sources of exposure, drinking water or being in contact with potting compost (table 11). As men and women showed different water-drinking behaviour, we give the data separately for men and women.

No statistically significant association between titers and drinking tab water are seen in table 11. Some higher titers are seen in those drinking water, especially at the toilettes, but as drinking water is associated with working in hall 3 or 4, these association are most likely due to confounding.

Table 11: Geometric mean titers (from first or only bloodsample) by drinking water and exposure to potting compost.

	IgG		IgM	
	yes (n)	no	yes (n)	no
Men (n=403)				
Drinking water from tab	21.2 (146)	20.8	15.8 (146)	14.7
- in toilettes	24.4 (62)	20.5	19.0 (62)	15.9
- in restaurants	18.3 (25)	21.1	13.4 (25)	15.2
- elsewhere	24.4 (81)	20.3	16.1 (81)	14.9
- water reservoir	31.6 (23)	20.5	13.7 (23)	15.2
Potting compost	19.6 (75)	22.0	16.1 (75)	15.3
Women (n=336)				
Drinking water from tab	21.4 (193)	19.9	19.6 (193)	16.5
- in toilettes	22.8 (92)	20.0	19.8 (92)	17.6
- in restaurants	19.7 (29)	20.9	18.3 (29)	21.1
- elsewhere	18.0 (89)	18.3	16.1 (89)	14.9
- water reservoir	20.4 (32)	20.8	16.9 (32)	18.4
Potting compost	17.7 (14)	20.5	12.9 (14)	18.4

4.5 Percentiles for antibody levels in reference sample

In figure 7 the age dependency in the mean titers from the serumbank sample is shown, by adding a LOESS-curve (LOESS is an statistical technique for smoothing data, and might be regarded as an (improved version of) a moving average).

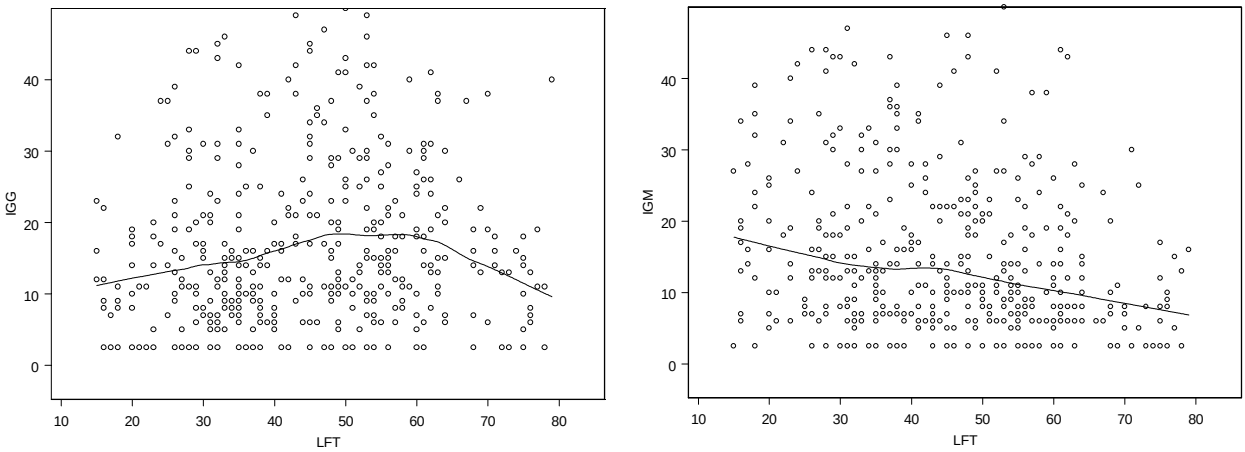

Figure 7: Age-dependency in titers for the serumbank sample: smoothed IgG (right) and IgM (left) antibody levels.

IgM antibodies decrease with age (p-value for a test of linear trend is 0.0001). For IgG titers increase with age until age 60, where after they decrease.

We can model the mean log titer as a function of age by fractional polynomials[24], yielding the following formula's:

Mean ln(IgG)=343.2746-0.0173*AGE**2+7.0936*AGE-86.2692*sqrt(AGE)-2282.2633/AGE+9632.8186/AGE**2

Mean ln(IgM)=60.0028-54.4298*ln(AGE)-1.0275*AGE+29.3483*sqrt(AGE)-1934.767/(AGE**2)

where AGE is age in years. Furthermore, the standard deviation of ln(IgM) around this mean is 0.8233974 and that of ln(IgG) is 0.9654902. Both this standard deviation and the skewness do not seem to be age dependent, and thus were not modeled as a function of age. Moreover, skewness did not statistically significantly differ from 0.

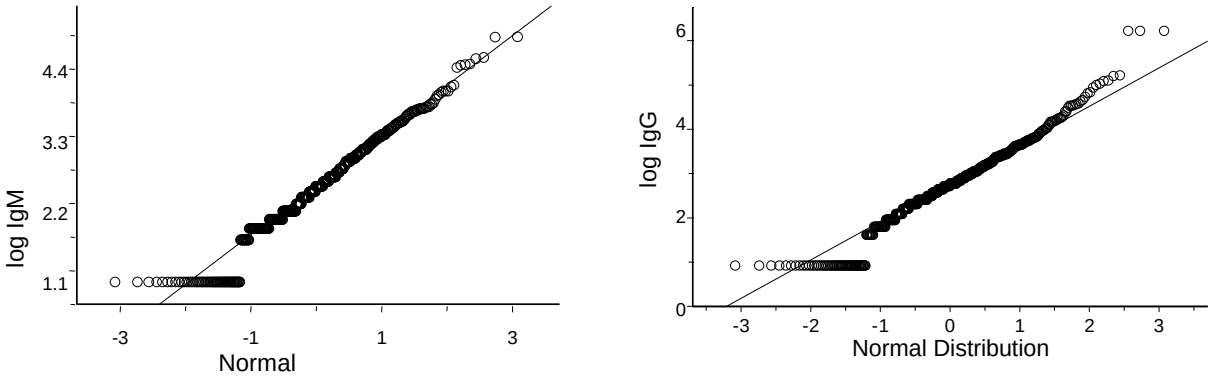

Figure 8: QQ-plots of the log antibody titers (ELISA) for the serumbank population. Left: IgM; right: IgG

From these results age-dependent percentiles can be calculated using skewness=0. This assumes that the distribution of titers is lognormal. QQ-plots of the data (figure 8), however, show that this assumption seems reasonable for IgM (apart from the aberration due to the presence of a detection threshold of 5 U/ml), but that the upper tail is too stretched out for IgG. In other words, compared to the (expected) lognormal distribution, the data contain to much persons with very high ($\approx >$ exp(4.5) = 90 U/ml) antibody levels. The result of this is that when we use the formulae given above, 1.5% (in stead of the expected 1%) of the subjects has an antibody titer above the 99th percentile. As this difference is small and not statistically, we keep the definition as given above, also because those with extreme, outlying values in the serumbank population might reflect the presence of a subgroup which is correctly positive. In table 12 we give the age-dependent 95^{th} and 99^{th} percentiles as calculated from the formulae given above. In table 13 the 95^{th} percentiles are compared to those calculated directly from the crude data in each age group. Direct estimates are not very precise, as for instance in the first row (16-24 years of age) the height of the percentile depends fully on the values for the second and third highest measurement, which are in such small sample sizes heavily influenced by random variation. In most strata the crude percentiles are similar to the model-based percentiles. Where there are differences, the model-based percentiles are somewhat higher. Furthermore these tables show that the cut-off points given by the manufacturer of the tests are roughly equal to the 95^{th} percentile for IgG, but are above the 99^{th} percentile for IgM.

Table 12: 95th and 99th age-dependent percentiles of the ELISA antibody tests from the model described in the text.

	IgG		IgM	
	95th percentile	99th percentile	95th percentile	99th percentile
16-24	55	106	65	114
25-34	68	131	54	95
35-44	73	141	48	85
45-54	90	174	45	78
55-64	99	192	38	67
65-74	53	102	25	44

Table 13: 95th age-dependent percentiles of the ELISA antibody tests: model data compared to crude figures.

	IgG		IgM	
	Model-based	Crude	Model-based	crude
16-24 (n=41)	55	55	65	40
25-34 (n=93)	68	68	54	47
35-44 (n=92)	73	70	48	43
45-54 (n=112)	90	74	45	46
55-64 (n=102)	99	96	38	38
65-79 (n=41)	53	38	25	24

In the serumbank sample 2 of the 481 samples yielded a micro-agglutination titer against Lp1 of 1:32, all other results were <1:32. From this, 1:32 can be seen as exceeding the 99th percentile. Titers against Lp6 were 1:256 for two cases, 1:64 for one case and 1:32 for 5 cases. From this, we regard 1:64 as exceeding the 99th percentile, and 1:32 as exceeding the 95th percentile.
When using the percentiles calculated as described above, defining a serological case as someone above the 99th percentile of at least 1 test (excluding the agglutination test for serogroup 6), the serum bank sample contains 2.9% of such serological cases. When we do not take IgG into account, this is 1.6%.

4.6 Serological cases by hall and by stand

Table 14 gives the serological cases by main place of work, both for all respondents and for those with complete information (= two blood samples tested, both with ELISA and micro-agglutination). Table 15 gives the same information, but this time using a case definition that only takes IgM titers (both ELISA and micro-agglutination) into account. The distribution of the probable cases over the halls differs significantly from that of the non-cases in all four

comparisons, but the distribution of possible cases only differs statistically significant from that of the non-cases for the first comparison in table 15 (p=0.02).
In figure 9 the probable, possible and non-cases are plotted on the map of the consumer-products fair (hall 3 and 4). As several persons worked at a single stand, plotting each person at the center of each stand would – because of overlapping symbols - not yield a meaningful picture. Therefore, the cases were jittered around the center of the stand, meaning that they were plotted at a small random distance from the center of their stand. Due to the jittering cases might in a few cases be plotted in a neighboring stand. These figures again show a larger number of probable and possible cases in hall 3, but it is difficult to see whether they are concentrated at a particular place within hall 3, although relatively less cases seem to be present at the left side of the hall. This confirms the earlier results in 4.3.

Table 14: Attack rates (in %) of serological cases by main place of work.

	Entire population (n=772)			Subjects with complete information (n=335)		
	Probable + possible cases	Probable cases	Possible cases	Probable + possible cases	Probable cases	Possible cases
Main workplace in hall 3	40.4	25.8	14.6	47.4	31.6	15.8
Main workplace in hall 4	14.8	7.2	7.6	21.6	12.5	9.1
Main work place at agricultural fair	23.6	10.6	13.0	38.4	17.2	21.2
Main workplace at flower show	17.2	8.6	8.6	17.4	4.4	13.0
Other workplace	16.3	4.7	11.6	20.0	5.7	14.3
Worked at multiple places	14.6	4.2	10.4	15.1	3.0	12.1
All	22.8	11.7	11.1	29.8	14.9	14.9

Table 15: Attack rates of serological cases by main place of work, when the case definition is based only on IgM (IgG ignored).

	Entire population (n=772)			Subjects with complete information (n=335)		
	Probable + possible cases	Probable cases	Possible cases	Probable + possible cases	Probable cases	Possible cases
Main workplace in hall 3	30.5	16.6	13.9	42.1	26.3	15.8
Main workplace in hall 4	8.0	3.2	4.8	11.4	5.7	5.7
Main work place at agricultural fair	15.5	6.1	9.4	27.3	11.1	16.2
Main workplace at flower show	17.1	5.7	11.4	10.1	4.4	5.7
Other workplace	11.7	4.7	7.0	14.3	5.7	8.6
Worked at multiple places	12.5	4.2	8.3	15.1	3.0	12.1
All	15.7	7.0	8.7	22.4	10.5	11.9

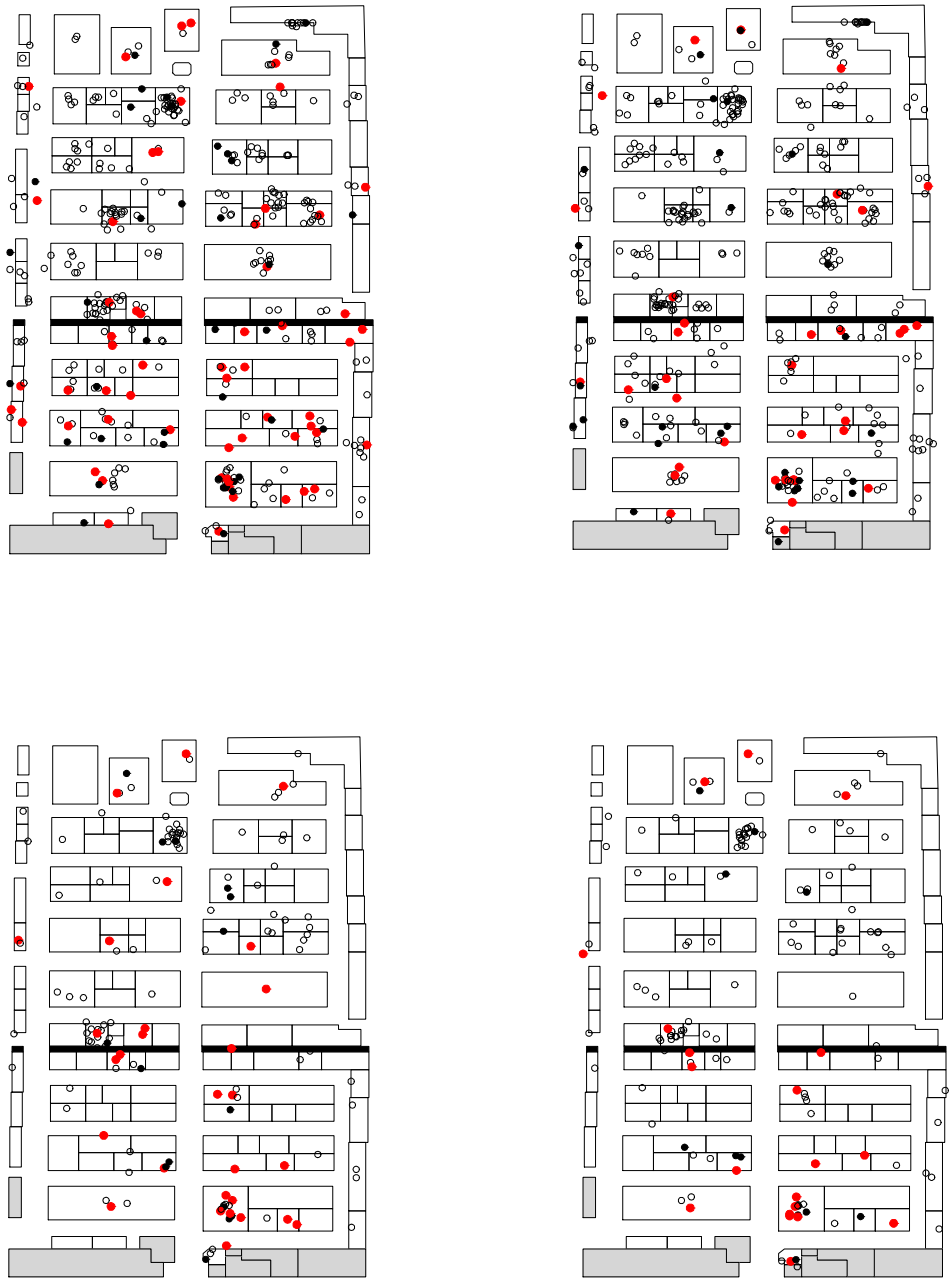

Figure 9: Probable serological cases (red dots), possible serological cases (black dots) and non serological cases (unfilled circles) per stand. The cases are jittered (see text) to prevent overlapping symbols. Left: Case definition based on both IgG and IgM. Right: case-definition based on IgM only. Upper figures: all subjects included. Lower figures: only subjects with complete information included.

5. Health complaints

5.1 Individual symptoms

Table 16 shows the total number of symptoms reported by the respondents. Like in the former analysis only those cases were included from whom questionnaire data were available and at least one blood sample was tested. The clinical symptoms were corrected for first day of illness i.e. only symptoms were included which started no earlier than February 23rd and no later than 19 days after the last visit to the Flora. Overall, the number of persons with symptoms was 542 (70%), but the number of persons with symptoms starting within 19 days of being on the Flora was 301 (39%). Headache, tiredness, having a cold, fever and diarrhea were most frequently reported. Of those who reported fever the mean highest measured temperature was 39.1 ºC (range 38.0-40.8 ºC).

Except for pneumonia, no other severe symptoms were reported. One man, working in hall 3, reported that pneumonia was confirmed and the physician suspecting Legionellosis. Although ELISA antibody titers (IgM and IgG) were not elevated, a positive result(1:32) was seen on the agglutination test for Lp1. Six persons reported that the physician suspected pneumonia and for 3 of them Legionellosis was thought of. Only one of them (where Legionellosis was suspected), working in hall 8, had somewhat elevated antibody levels (ELISA IgM titer between 95th and 99th percentile). Another 8 persons reported that the physician suspected Legionellosis but no suspected pneumonia was reported. Two of them, both from hall 3, had ELISA antibody titers above the 99th percentile, in the others no elevated titers were present. One other person, not included in the table, reported that the physician suspected Legionellosis. However, this person indicated that symptoms had started on the 20th, and led to work absenteeism on the 22nd, although they became severe enough to warrant staying in bed only on the first of March. No increased antibody levels were seen in this person.
Overall, females reported symptoms more frequently than males. Among persons <50 years compared with persons >= 50 years, headache (20% vs. 10%) was more frequently reported in the youngest age group. Also the proportion of persons with one ore more symptoms was higher among the younger age group (<50 years) compared to the older age group (42% vs. 34%).

Table 16: Frequency of clinical symptoms which started between February, 23rd and 19 days after the last visit to the Flora.

Symptoms	Total (n= 772)	Mean duration (days) (range)	Gender†	
			Male (n=419)	Female (n=346)
Dry cough	63 (8.2%)	15.5 (2-49)	32 (7.6%)	31 (9.0%)
Productive cough	59 (7.6%)	17.8 (3-43)	29 (6.9%)	29 (8.4%)
Shivering	82 (10.6%)	7.9 (1-49)	36 (8.6%)	44 (12.7%)
Muscular pain	62 (8.0%)	12.3 (1-56)	32(7.6%)	30 (8.7%)
A cold	118 (15.3%)	14.1 (1-49)	58 (13.8%)	60 (17.3%)
Headache	136 (17.6%)	11.3 (1-52)	53 (12.7%)	81 (23.4%)**
Earache	21 (2.7%)	13.3 (1-36)	8 (1.9%)	12(3.5%)
Diarrhea	88 (11.4%)	7.8 (1-37)	34 (8.1%)	53 (15.3%)*
Dizziness	55 (7.1%)	11.7 (1-34)	18(4.3%)	36 (10.4%)**
Nausea	54 (7.0%)	7.6 (1-37)	15 (3.6%)	39 (11.3%)**
Vomiting	32 (4.1%)	5.0 (1-30)	13 (3.1%)	19 (5.5%)
Listlessness/apathy	64(8.3%)	17.9 (2-66)	26 (6.2%)	36 (10.4%)*
Tiredness	134 (17.4%)	17.1 (1-69)	57 (13.6%)	75 (21.7%)**
Confusion	15 (1.9%)	10.9 (1-34)	4 (1.0%)	9 (2.6%)
Abdominal pain	37 (4.8%)	11.9 (1-34)	11 (2.6%)	26 (7.5%)**
Shortness of breath	40 (5.2%)	17.8 (1-38)	11 (2.6%)	28 (8.1%)**
Wheezy breathing	23 (3.0%)	20.9 (2-40)	7 (1.7%)	15 (4.3%)*
Painful breathing	20 (2.6%)	10.8 (1-22)	3 (0.7%)	17 (4.9%)**
Heart palpitations	17 (2.2%)	11.4 (1-33)	5 (1.2%)	11 (3.2%)
Lack of appetite	46 (6.0%)	11.3 (1-52)	18 (4.3%)	27 (7.8%)*
Rash	14 (1.8%)	19.2 (1-35)	6 (1.4%)	8 (2.3%)
Stomach ache	19 (2.5%)	15.7 (1-43)	6 (1.4%)	13 (3.8%)*
Other symptoms	37 (4.8%)	7.6 (1-34)	12 (2.9%)	25 (7.2%)**
No symptoms	471(61.0%)	--	273 (65.2%)	194 (56.1%)*
Fever (range temp.)	91 (11.8%)	39.1(38.0-40.8)	33 (7.9%)	50 (14.5%)*
Pneumonia confirmed	1 (0.1%)	--	1 (0.3%)	--
Pneumonia suspected	6 (0.8%)	--	2 (0.5%)	4 (1.2%)
Legionellosis suspected	12 (1.6%)	--	3 (0.7%)	7 (2.2%)

* Chi-square test or (if n<6) Fisher's exact test: p<0.05 ** chi-square test <0.005

† gender is missing for 7 persons

Due to illness, 10% stayed in bed and 9% were absent from work (table 17) in the 19 days after being on the Flora. Nineteen percent of the respondents consulted a physician of whom more women than men. Counting only those who also indicated symptoms within 19 days after being on the Flora, this was fourteen percent. 4 persons were hospitalized although no pneumonia or Legionella was confirmed or suspected, but none were hospitalized within a 19-day period after being on the Flora. Six percent reported that their housemates who did not visit the Flora had comparable symptoms, but including only those with symptoms within 19 days after being at the Flora, this was only 4 percent. Having such housemates with comparable symptoms might indicate that the reported symptoms were not related to their visit to the Flora.

Table 17: Impact of clinical symptoms (bed rest / absence or hospitalization within 19 days after the last visit to the Flora).

Symptoms	Total (n=772)	Mean duration days (range)	Gender†	
			Male (n=419)	Female (n=346)
Ill in bed	78 (10.1%)	4.0 (1-18)	37 (8.8%)	40 (11.6%)
Absence due to illness	68 (8.8%)	5.6 (1-20)	40 (9.5%)	27 (7.8%)
Physician consulted ‡	111 (14.4%)	--	47 (11.3%)	62 (18.7%)*
Hospitalization	0 (0.0%)	--	0 (0.0%)	0 (0.0%)
Taking antibiotics ‡	65 (8.4%)	--	29 (6.9%)	34 (9.8%)
Housemate comparable symptoms ‡	26 (3.5%)	--	15 (3.7%)	11 (3.4%)

* Chi-square test P<0.05
† gender is missing for 7 persons
‡ only those with symptoms within 19 days after visiting the Flora.

Table 18 shows the distribution of clinical symptoms by main workplace. Persons who had been working on the flower show reported having no symptoms most frequently. Overall, no great differences in reported symptoms were observed between different main work places. If hall 3 is compared to all other main work places, we see some statistically significant differences (dry cough, muscular pain, earache, listlessness/apathy and stomach-ache) in individual complaints, as well as in the total number of persons with complaints. When we adjust the analysis for age, sex, smoking, alcohol use, pre-existing lung disorders and immunosuppressive disorders/medication, results do not change, although the prevalence of muscular pain no longer is statistically significantly different in hall 3 compared to the other halls together. If we exclude respondents who have housemates with comparable symptoms, results are similar, only in the unadjusted analyses (but not in the adjusted analyses) now also dizziness and confusion show a statistically significant difference between hall 3 and the

other halls. In these analyses (excluding those with housemates with comparable symptoms) muscular pain remains statistically significantly increased in hall 3 in the multivariate analysis. No differences between hall 3 and the other halls are seen in the frequency of fever, sickness absenteeism, visiting a physician or staying in bed because of illness.

Table 18: Frequency of symptoms stratified by hall (n=772).

	Main workplace hall 3 (n=151)	Main workplace hall 4 (n=249)	Main workplace agricultural fair (n=246)	Main workplace flower show (n=35)	Other workplaces (n=43)	Worked at multiple locations (n=48)
Dry cough	19 (12.6%)†	17 (6.8%)	20 (8.1%)	1 (2.9%)	1 (2.3%)	5 (10.4%)
Productive cough	12 (8.0%)	19 (7.6%)	15 (6.1%)	1 (2.9%)	5 (11.6%)	7 (14.6%)
Shivering*	17 (11.3%)	32 (12.9%)	19 (7.7%)	0 (0%)	5 (11.6%)	9 (18.8%)
Muscular pain	18 (11.9%)†	20 (8.0%)	16 (6.5%)	1 (2.9%)	3 (7.0%)	4 (8.3%)
A cold	30 (19.9%)	35 (14.1%)	34 (13.8%)	2 (5.7%)	8 (16.6%)	9 (18.8%)
Headache	30 (19.9%)	43 (17.3%)	41 (16.7%)	3 (8.6%)	11 (25.6)	8 (16.7%)
Earache	9 (6.0%)†	7 (2.8%)	2 (0.8%)	1 (2.9%)	0 (0%)	2 (4.2%)
Diarrhea	19 (12.6%)	33 (13.3%)	24 (9.8%)	1 (2.9%)	5 (11.6%)	6 (12.5%)
Dizziness	16 (10.6%)	20 (8.0%)	14 (5.7%)	0 (0%)	1 (2.9%)	4 (8.3%)
Nausea	13 (8.6%)	21 (8.4%)	11 (4.5%)	0 (0%)	6 (14.0%)	3 (6.3%)
Vomiting	7 (4.6%)	11 (4.4%)	10 (4.1%)	0 (0%)	2 (4.7%)	2 (4.2%)
Listlessness/apathy*	23 (15.2%)††	23 (9.2%)	10 (4.1%)	2 (5.7%)	2 (4.7%)	4 (8.3%)
Tiredness	30 (19.9%)	49 (19.7%)	32 (13.0%)	3 (8.6%)	8 (18.6%)	12 (25.0%)
Confusion	6 (4.0%)	7 (2.8%)	1 (0.4%)	0 (0%)	0 (0%)	1 (2.1%)
Abdominal pain	8 (5.3%)	17 (6.8%)	6 (2.4%)	0 (0%)	1 (2.3%)	5 (10.4%)
Shortness of breath	9 (6.0%)	16 (6.4%)	10 (4.1%)	0 (0%)	2 (4.7%)	3 (6.3%)
Wheezy breathing	4 (2.7%)	12 (4.8%)	5 (2.0%)	0 (0%)	0(0%)	2 (4.2%)
Painful breathing	7 (4.6%)	5 (2.0%)	6 (2.4%)	0 (0%)	1 (2.3%)	1 (2.1%)
Heart palpitations	4 (2.7%)	6 (2.4%)	4 (1.6%)	0 (0%)	0 (0%)	3 (6.3%)
Lack of appetite*	13 (8.6%)	19 (4.8%)	4 (1.6%)	2 (5.7%)	2(4.7%)	6 (12.5%)
Rash	5 (3.0%)	19 (7.6%)	4 (1.6%)	2 (5.7%)	2(4.7%)	2 (4.2%)
Stomach ache	8 (5.3%)†	6 (2.4%)	2 (0.8%)	0 (0%)	2 (4.7%)	1 (2.1%)
Other symptoms	10 (6.6%)	14 (5.6%)	10 (4.1%)	0 (0%)	1 (2.3%)	2 (4.2%)
No symptoms*	79 (52%)†	152 (61%)	157 (56%)	29(83%)	27 (63%)	27 (56%)
Fever	19 (12.6%)	31 (12.5%)	31(12.6%)	1 (2.9%)	4 (9.3%)	5 (10.4%)
Taking antibiotics	14 (9.4%)	16 (6.6%)	11 (4.5%)	0 (0)	2 (4.8%)	5 (10.4%)

* statistically significant differences between halls.
† $p<0.05$ for comparison of hall 3 with all other halls
†† $p<0.005$ for comparison of hall 3 with all other halls

Table 19: Frequency of symptoms of serological cases.

Symptoms	Entire population (n=772)			Correlation with antibody titers	
	No (n=596)	Possible (n=86)	Probable (n=90)	ln IgG (in first or only sample)	ln IgM (in first or only sample)
Dry cough	53 (8.9%)	6 (7.0%)	4 (4.4%)		
Productive cough	46 (7.7%)	7 (8.1%)	6 (6.7%)		
Shivering	61 (10.2%)	10 (11.6%)	11 (12.2%)	*	
Muscular pain	43 (7.2%)	8 (9.3%)	11 (12.2%)	**	
A cold	96 (16.1%)	12 (14.0%)	10 (11.1%)		
Headache	108 (18.1%)	13 (15.1%)	15 (16.7%)		*
Earache	17 (2.9%)	1 (1.2%)	3 (3.3%)		
Diarrhea	73 (12.3%)	8 (9.3%)	7 (7.8%)		
Dizziness	43 (7.2%)	5 (5.8%)	7 (7.8%)		
Nausea	39 (6.5%)	10 (11.6%)	5 (5.6%)		
Vomiting	24 (4.0%)	6 (7.0%)	2 (2.2%)		
Listlessness/apathy	49 (8.2%)	6 (7.0%)	9 (10.0%)		
Tiredness	104 (17.5%)	15 (17.4%)	15 (16.7%)		
Confusion	13 (2.2%)	0 (0%)	2 (2.2%)		(*)
Abdominal pain	31 (5.2%)	1 (1.2%)	5 (5.6%)		
Shortness of breath	32 (5.4%)	4 (4.7%)	4 (4.4%)		
Wheezy breathing	21 (3.5%)	1 (1.2%)	1 (1.1%)		
Painful breathing	17 (2.9%)	1 (1.2%)	2 (2.2%)		
Heart palpitations	13 (2.2%)	1 (1.2%)	3 (3.3%)		
Lack of appetite*	36 (6.0%)	4 (4.7%)	6 (6.7%)		
Rash	13 (2.2%)	1 (1.2%)	0 (0%)		
Stomach ache	13 (2.2%)	1 (1.2%)	5 (5.6%)	*	
Other symptoms	26 (4.4%)	4 (4.7%)	7 (7.8%)		
No symptoms	359 (60.2%)	52 (60.5%)	60 (66.7%)		
Fever	71 (11.9%)	9 (10.4%)	11 (12.2%)		

* $p<0.05$; (*): $P<0.05$, but association in the wrong direction (more symptoms associated with lower titer)
** $p<0.005$

In table 19 we classified the respondents according to the case definition as defined in paragraph 2.5.1.4. Looking at the frequency of symptoms there are no statistical significant

differences between the groups of serological cases. When we adjust the analysis for age, sex, smoking, alcohol use, pre-existing lung disorders and immunosuppressive disorders/medication still no statistically significant differences are seen between the probable/possible cases in any complaint. For a few complaints there are, mostly weak, relationships with the IgM or IgG antibody titers. When we adjust the analysis for age, sex, smoking, alcohol use, pre-existing lung disorders and immunosuppressive disorders/medication, all the relations observed univariately remain statistically significant with the exception of that between IgM and headache.

5.2 Clinical illness

Loosely following Bell et al.[3], we defined clinical illness compatible with legionellosis as illness characterized by fever or two or more of the following symptoms: cough, shortness of breath, painful breathing, muscle pain, headache, dizziness, diarrhea, vomiting, stomachache, confusion, listlessness, shivering. We also included persons of whom the physician suspected pneumonia or LD. In table 20 we give the frequency of such illness by hall, and also according to whether the person was a possible or probable serological case. There were no statistically significant differences seen between all halls, nor between hall 3 (25.2%) and the other halls (21.6%), neither univariately nor when adjusting for age and gender. Also after excluding those with a housemate suffering from similar symptoms no such relations were seen. There is even a tendency in those with higher antibody titers towards a lower prevalence of legionellosis compatible symptoms.

Table 20: Frequency of clinical illness compatible with legionellosis and absolute number of cases by hall and serological status (n=772).

	All	Possible or probable serological case	
		No	Yes
	% (n)	% (n)	% (n)
Main workplace hall 3	25.2 (38)	27.8 (25)	21.3 (13)
Main workplace hall 4	23.3 (58)	23.1 (49)	24.3 (9)
Main workplace agricultural fair	21.1 (52)	22.3 (42)	17.2 (10)
Main workplace flower show	5.7 (2)	3.5 (1)	16.7 (1)
Other workplaces	23.3 (10)	25.0 (9)	14.3 (1)
Worked at multiple locations	25.0 (12)	24.4 (10)	28.6 (2)
All	22.3 (172)	22.8 (136)	20.5 (36)

6. Attack rates

6.1 Attack rates of Legionnaire's disease in the exhibitors

In order to estimate the attack rate in the exhibitors, we tried to locate all cases with Legionnaire's Disease (LD) in the exhibitors and other persons professionally present at the Flora.
We used 4 sources to locate these cases:

- The case-register. In the case-register of the epidemic it was noted whether a case was an exhibitor or not. For many cases, however, this information was not available
- The case-control study. In the case-control study subjects were asked whether they were a visitor or an exhibitor.
- The mailing list of our study. In the mailing list it was sometimes noted that some-one was hospitalized, or only sent a questionnaire because "blood had already been investigated". We checked whether these persons were in the case register.
- The results of the questionnaire: all persons who indicated that they either had had pneumonia or Legionnaire's Disease, or that their physician had suspected this, where checked against the case-register.

We follow the definition of confirmed and probable cases of LD as used before in the case-control study and the case register (see section 2.2).

This yielded 13 cases from the case register who were professionally present at the Flora. Table 21 gives the data available from these cases.

Table 21: Information on cases of Legionnaire's disease in exhibitors.

	Confirmed (9)/ Probable(1)			Others(3)		
	Total non-response	Only questionnaire data	Questionnaire and blood sample	Total non-response	Only questionnaire data	Questionnaire and blood sample
Not approached	4			2		
Sent only questionnaire		2 *				1**
Approached	2	1	1			

* One of these was the probable case;
**Was sent a request for the second blood sample only, and thus only a single blood sample was analyzed

The case register of the epidemic comprised 13 persons who were known to be an exhibitor. 9 of those were confirmed cases, 1 a probable case. The confirmed cases had worked on stands 3.04, 3.11, 3.21 (two cases), 3.35, 3.36, 3.55, 4.14 and 4.60. The one probable case worked at 3.11.
For the 3 cases that were not confirmed/probable cases, information is only available for the case that participated in the cohort study (but was excluded in the analysis because of possible LD). This person reported to have been diagnosed for certain with LD and worked at stand 3.32. This case had high antibody levels, but did not have a clinical diagnosis of pneumonia, only influenza like symptoms including high fever.

From these data we estimated the attack rates, both per hall, and by distance from the whirlpool in hall 3. In order to calculate attack rates, one needs to estimate the population at risk. We used our mailing list as enumerator, after adding the 6 LD cases that were not on the mailing list. The numbers on the mailing list can only be seen as an approximation, as the non-response forms showed that there were persons on the mailing list who did not work on the fair. On the other hand, not all firms who were present on the show were reached, and the spontaneous reaction after the first mailing showed that not all names of persons who worked on the stands were given by the firms. Nevertheless, we think it is a fair estimate. Especially the number of firms not reached was small, as only two stands in hall 8 and one in hall 4 were not on the mailing list. As before, we excluded those working at stand 3.03 and 3.13 from hall 3.
The attack rates calculated by hall and by distance to the whirlpool in hall 3 are shown in table 22.

There is a clear gradient with the distance to the whirlpool in hall 3 ($p=0.0009$). Also the attack rate in hall 3 is higher than that in hall 4 (exact p-value 0.005), as well as in all other halls (exact $p <0.0001$).

Table 22: Attack rates of confirmed/probable LD.

	Estimated number at risk	Confirmed/probable cases of LD	Attack rate
All exhibitors	1670	10	0.6 %
Hall 3	291	8	2.8 %
Hall 4	541	2	0.4 %
Hall 8	626	0	0 %
Other/unknown	212	0	0 %
Distance to the whirlpool in hall 3*			
< 15 meter	115	5	4.3 %
15-30 meter	67	2	3.0 %
30-60 meter	103	2	1.9 %
60-90 meter	284	1	0.4%
>90 meter	105	0	0 %

* Only persons working in hall 3 and 4 are included

6.2 Attack rates of serological cases

Attack rates of serological cases by hall are already given in table 14 and table 15.
In table 23 we present the attack rates by distance to the whirlpool in hall 3.
There is a clear decrease in the attack rate with increasing distance to the whirlpool in hall 3. (p-values from 0.002 to <0.001). However, there is no significant difference in the attack rates within 15 meters from this whirlpool and within 15 to 30 meters from this whirlpool. However, the difference of the attack rates within 30 meters in most cases is statistically significantly higher than the attack rate within 30-60 meters. The maximum distance for persons in hall 3 is 54.7 meter, while the minimum distance for persons working in hall 4 is 44.7 meters. So it should be remembered that the contrast seen reflects at least partly the contrast between hall 3 and hall 4.
We carried out a similar multivariate logistic regression, in order to adjust for confounding variables. Results are shown in table 24. For comparison purposes also the odds ratio's calculated from the crude data in table 23 are given.

Table 23: Attack rates based on serological cases by distance from the whirlpool in hall 3 (only respondents working in hall 3 or 4).

	N	Case definition based on both IgG and IgM				Case definition based on IgM only			
		Probable cases		Probable + possible cases		probable cases		Probable + possible cases	
		Attack rate (%)	Relative risk	attack rate (%)	relative risk	attack rate (%)	relative risk	attack rate (%)	relative risk
All respondents (n=384)									
0-15 m	57	29.8	3.6 [1.88-7.0]	45.6	2.7 [1.77-4.4]	17.5	3.5 [1.44-8.3]	35.1	3.3 [1.84-5.8]
15-30 m	22	27.3	3.3 [1.41-8.7]	45.5	2.8 [1.55-4.9]	18.2	3.6 [1.18-10.9]	31.8	3.0 [1.39-6.3]
30-60 m	147	15.7	1.90 [1.00-3.6]	24.5	1.49 [0.95-2.3]	8.8	1.75 [0.75-4.1]	15.0	1.39 [0.77-2.5]
>60 m	158	8.2	1.0	16.5	1.0	5.1	1.0	10.8	1.0
p-value trend			<0.001		<0.001		0.002		<0.001
Respondents with complete information only (n=139)									
0-15 m	25	40.0	4.6 [1.74-12.0]	56.0	3.2 [1.65-6.2]	36.0	5.1 [1.74-15.1]	56.0	4.0 [1.92-8.3]
15-30 m	13	30.8	3.5 [1.09-11.3]	53.9	3.1 [1.44-6.5]	23.1	3.3 [0.84-12.9]	38.5	2.7 [1.07-7.0]
30-60 m	44	22.7	2.6 [0.95-7.0]	29.6	1.68 [0.82-3.5]	9.1	1.30 [0.34-4.9]	11.4	0.81 [0.29-2.3]
>60 m	57	8.8	1.0	17.5	1.0	7.0	1.0	14.0	1.0
p-value trend			<0.001		<0.001		<0.001		<0.001

Table 24: Odds ratio's for attack rates by distance to the whirlpool in hall 3 only respondents working in hall 3). Unadjusted and adjusted for age, gender, immunosuppressive disorders/medication, lung disorders, smoking and alcohol use.

	Probable cases		Possible and probable cases	
Case definition based on both IgG and IgM				
	Unadjusted	Adjusted	Unadjusted	adjusted
0-15 m	4.7 [2.1 –10.5]	4.3 [1.83-10.3]	4.3 [2.2-8.3]	4.6 [2.2-9.7]
15-30 m	4.2 [1.40-12.5]	4.4 [1.25 –14.3]	4.2 [1.66–10.9]	4.2 [1.45-11.9]
30-60 m	2.1 [1.01-4.3]	2.0 [0.96 –4.4]	1.65[0.94-2.9]	1.52 [0.84-2.8]
>60 m	1.0	1.0	1.0	1.0
Case definition based on IgM only				
0-15 m	4.0[1.49-10.6]	4.3 [1.48-13.2]	4.5 [2.1-9.4]	5.4 [2.4-12.3]
15-30 m	4.2 [1.14-15.2]	4.2 [0.83-17.7]	3.9 [1.38-10.9]	4.4 [1.33-13.7]
30-60 m	1.82 [0.73-4.5]	2.1 [0.79-5.7]	1.46 [0.74-2.9]	1.44 [0.70-3.0]
>60 m	1.0	1.0	1.0	1.0

6.3 Attack rates of infection

We used Bayesian methods (see appendix 3) to estimate the probability of infection in those working at the consumer products fair, taking both their own antibody levels, and those of persons working close to them into account. The probability of infection is estimated by comparing the antibody level with the distribution of antibody levels observed in the serumbank sample. However, it is possible that the antibody distribution in the reference sample is not representative for the distribution in the exhibitors before coming to the Flora, e.g. because of the preceding influenza epidemic, or because exhibitors tend to have higher antibody levels than the general population for other reasons. Therefore we also carried out these calculations using the exhibitors working in hall 8 as reference sample.

A surprisingly high figure of persons infected was calculated for hall 3 using both reference groups (table 25). This means that the data are most compatible with many persons being infected, while such an infection increases the antibody levels only moderately. Not surprisingly, the same relation with distance from the whirlpool is seen as in other analyses.

Table 25: Average probability of infection as estimated by the Bayesean model (appendix 3), when using the serum bank titers as the titer distribution of unexposed, and when using the exhibitors in hall 8 as such.

	With serumbank as reference	With exhibitors in hall 8 as reference
Work place:		
Hall 3	80.0%	70.0%
Hall 4	24.8%	19.8%
Distance to the whirlpool in hall 3		
< 15 meter	85.2%	80.8%
15-30 meter	78.7%	66.5%
30-60 meter	44.5%	35.5 %
60-90 meter	25.9%	21.1%

6.4 Attack rates of health complaints

Table 26 shows the clinical cases by distance to the workplace in hall 3. We defined clinical illness compatible with legionellosis in paragraph 5.2. We classified subjects both according to the presence or absence of clinical illness compatible with legionellosis, and according to whether they had such clinical illness in combination with being a serological possible/probable cases. Table 26 and table 20 both show that there are no statistically significant differences between groups and in distance to the whirlpool in hall 3. Multivariate logistic regression was used to adjust for age, gender and the other potentially confounding factors. Results did not show any statistically significant relation with the distance to the whirlpool in hall 3 either.

Table 26. Attack rates (in %) of clinical cases by distance from the whirlpool in hall 3 (only respondents working in hall 3 or 4).

N		Clinical symptoms		Clinical symptoms and being a possible/probable serological cases		
All respondents	n	Attack rate(n)	Relative risk	n	Attack rate(n)	Relative risk
0-15 m	61	24.6% (15)	1.1 (0.6-1.8)	57	7.0 (4)	2.8 (0.7-11)
15-30 m	24	25.0% (6)	1.1 (0.5-2.4)	22	0 (0)	0 (-)
30-60 m	150	23.3% (35)	1.0 (0.7-1.6)	146	4.8 (7)	1.9 (0.6-6.3)
>60 m	160	22.5% (36)	1.0	157	2.5 (4)	1.0
P value trend		0.8			0.2	

6.5 Pontiac fever

Pontiac fever is diagnosed as influenza like symptoms combined with an increase in antibodies against Legionella. To study Pontiac fever-like cases we started with selecting those cases with an acute illness characterized by fever and one or more of the following symptoms: headache, cough or muscle pain. The incubation period had to be short with symptoms starting within 2 days after the last visit to the Flora. Only 22 persons met these first criteria for Pontiac fever-like illness. Eight were male (35%) and 14 (64%) were female with a median age of 45 years (range 11-64 years). The median measured temperature was 39.6 °C (range 38.9-40.8 °C). Four persons were according to the case definition based on IgM and IgG titers, ‘probable’ cases while 2 persons were ‘possible’ cases. Only 1 person with high IgM titers reported that the physician suspected both pneumonia and Legionnaire’s disease. This person is more likely to have been a case of (mild) LD rather than of Pontiac Fever.
With respect to exposure, 7 persons were mainly working in hall 3, 6 persons mainly in hall 4, another 6 persons mainly in the agricultural hall and the other 3 persons were working in various places.

7. Discussion

7.1 Study design

We will discuss several aspects of our study, in order to assess whether imperfections of the study might have cause distortion of the results.

7.1.1 Response

The response on the questionnaire was only 52%. This rather low response is partly due to the fact that the mailing list was assembled in a short time, and thus is likely to have contained names of persons who should not have been on the list, because they did not work at the Flora. If the non-response forms are representative for all non-responders in this respect, response of the real target population would be 62% rather than 52%. Furthermore, it is likely that a considerable part of the non-participation is due to unwillingness to have a blood sample taken, and it is not likely that this unwillingness will be related to either exposure or outcome of this study. Nevertheless, the non-response remains high enough to warrant a close look at the possibility of bias by selective non-response.
Main outcome measures are the magnitude of antibody titers and health complaints. When non-response would be increased in particular combinations of outcomes and exposure, this would bias results.
For one combination such selective non-response is possible: The non-response analysis indicates that those who had Legionellosis-like complaints and who also worked at the consumer products fair might be underrepresented. This means that the relation between health complaints and working in hall 3 might have been underestimated. It can be doubted, however, if this effect is very strong. In view of the very weak association between LD like health complaints and antibody titers, it is unlikely that such selective non-response will have caused any significant bias in the results concerning antibody titers.

7.1.2 Other missing data

The largest amount of missing data was generated because EDTA tubes were sent out in the first mailing in stead of coagulation tubes, and we therefore erroneously received plasma in stead of serum as a first sample in about half the participants. Thus no micro-agglutination tests could be carried out in those first samples. Fortunately, the ELISA test can also be carried out in plasma. Moreover, the micro-agglutination test seems to be less sensitive than the ELISA for detecting differences between groups. This problem, therefore, had only a limited influence on the results of the study.

More than hundred participants did not respond to the request for a second blood sample. However, analyses could be carried out also using just the first blood sample, and results were similar to results using only the participants for whom two blood tests were done. 5 cases could not be included in the analyses because their blood only sample was not usable for analyses and only 3 cases completed the questionnaire so badly that exposure information was inadequate. It is not likely that this will have caused major bias.

7.1.3 Misclassification of exposure or outcome

The questionnaire was completed more than one month after the end of the Flora, and therefore a participant might not remember all particulars of his/her whereabouts during the Flora. However, most of the time will have been spent at the stand where a person worked, and this place could be ascertained with reasonable certainty. Selective recall bias might be present in those who had experienced legionellosis-like health complaints; however – again in view of the low correlation between antibody titers and health complaints – it is not likely that this will have been related to the magnitude of the antibody titer.

The data collection of the study was organized and carried out in a very short time and under high time pressure. In order to guarantee as much anonymity as possible, blood samples were collected in tubes on which the only identifying information was the number assigned to the participant for this study. There are clear indications that in the logistic process some errors have been made, e.g. that there were instances where a tube with a certain number was sent to the wrong participant, and thus antibody results also are assigned to the wrong person. Nevertheless, it is unlikely that these errors will have been frequent. Moreover, in cases of errors the person probably will have been sent a tube with a number preceding or following one's own number. As the mailing list was compiled by employer, a preceding or following person would often have worked at the same stand. Also, it is unlikely that a similar error will have been made for both blood samples. This means that especially the data on seroconversions are vulnerable for these mismatches. However, we screened the data to see whether there were seroconversions which could be explained by such errors on persons with consecutive numbers and did not note any. We belief that the number of errors due to such mismatches is fairly limited. Nevertheless, in future studies which need to be carried out in a short time, it is recommendable to let the participant or the person who collects the blood sample add some identifying information (e.g. data of birth) to enable checking the correctness.

7.1.4 Timing of the study

As usual in an outbreak investigation, the study was conceived, designed and started in a very short period. Nevertheless, as the epidemic was only discovered almost two weeks after the

end of the Flora, and conceiving and organizing the study also took two weeks, the first blood samples were taken at a time when seroconversion will already have taken place in most participants. The amount of seroconversion observed in this study therefore was limited, and, in retrospect, the value of having a second blood sample taken is moot, especially as the absolute titers proved to yield good alternative information on the location of the *L. pneumophila* spreading source.

7.2 Discussion of results

A very prominent feature of our data are the increased antibody titers in all participants, also in those not from hall 3, in comparison to the serum bank sample.
Possible explanations for such an effect are:

- The increased antibody titers are due to a small boosting effect of exposure to *L pneumophila*, which is not large enough to be called a true antibody response. Such small responses to low doses have been observed for other agents. Moreover, in guinea pigs a dose response relation between the given dose of *L pneumophila*, and the level of antibodies in serum 4 weeks later has been observed[4].
- The increased antibody titers are due to cross-reactivity to another agent that was more prevalent at the time of the Flora than at the time the serum bank sample was collected. One could think of the fact that the Flora was held in the wake of the yearly influenza epidemic.
- The increased antibody titers are due to the fact that the exhibitors do not represent a random sample from the Dutch population. Because of their profession they might be more in contact with *L pneumophila* than the general population.
- The lower levels of antibodies measured in the serumbank sample are due to disintegration during storage in the serum bank. However, this is not thought to be very probable.

From a further analysis of the antibody data (appendix 3), we estimated that 80% of those working in hall 3 were ‘infected’ (judged by their antibody levels) on the Flora when using the serum bank sample as representing the antibody titers in those not ‘infected’. When the distribution of antibody levels in those working in hall 8 were used in stead as the reference, the percentage was 70%. This also suggests that exposure to *L pneumophila* on the Flora will have caused a slight antibody response in many, in stead of a large response in a few.
If this is indeed the case, determining antibody levels would be a more sensitive tool for source detection in outbreaks than expected beforehand.

The association between duration of exposure and antibody level was very weak. If any dose-effect relationship with duration is present, it seems to be limited roughly to the first 2 hours.

For IgG there was even an tendency to decrease with very long exposure times (> 10-20 hours).
There are several potential (and speculative) explanations for such an effect:

- Those with long exposures are the professional exhibitors, who might have been exposed to *L. pneumophila* before on other exhibitions. In general the IgM reaction is expected to be lower in secondary exposures, but boosting of IgG might still be expected. However, whether this is indeed the case for infections with *L. pneumophila* is unknown. Our data do not indicate such a differential effect.
- Selection: LD cases are excluded in this study. Possibly they had long exposure times, and therefore their exclusion causes a false leveling off of the antibody versus duration of exposure relation.
- Saturation: Exposure above a certain level does not matter. By this level one either will have a response, or one's immune system will not react, no matter how long the exposure.
- Duration of exposure is related to the probability of having been near the source. However, those who spent many hours on the Flora spent the additional hours purely as "working" hours at their own stand. The absolute amount of general walking around does not increase any more above a certain staying time

Health complaints were only very weakly related to high antibody titers. This suggests that the increases in antibody levels observed are mostly caused by successful elimination of (small) amounts of infective material by the immune system, and not by the occurrence of a mild form of LD. Nevertheless, for some symptoms an increase with antibody titer was seen. The difference in legionellosis resembling complaints between hall 3 and other halls is small (25.2% in hall 3 against 21.6% elsewhere) and not statistically significant. The difference in some individual mild symptoms (mostly occurring without fever) is larger between hall 3 and the other halls: for a dry cough e.g. the difference is 5.5%.
This means that it is possible that for each case of LD a few cases of such individual milder symptoms might have occurred, but that we can not demonstrate this with our data.

Only the hotspot for IgG in paired samples observed in hall 3 is situated over the whirlpool in hall 3. The hotspot for IgM is not centered either on the whirlpool or the bubble mat. This might be explained by air movements (e.g. due to the ventilators that were used) or movements of visitors within the hall. However, it should be remembered that the smoothing method might also cause some artifacts. In smoothing, the average is taken from the 35 nearest neighbors. Where data from exhibitors are sparse, the titers of few exhibitors will influence the average titers in a large area; where data are dense the influence of each exhibitor will be more local.
This is nicely illustrated by the stand of the Dutch Horticultural Society, in the upper middle part of hall 4, from which 28 exhibitors participated. This causes a circle of lighter color around this stand in the smoothed map. Also, the hot spot above the whirlpool in hall 3 in the IgG map extents to the space on the map below the whirlpool, while no data from exhibitors

from this space are present. Nevertheless, figure 4 and 9 also show that in the 3 exhibitors working in the stand with the whirlpool in hall 3 high antibody levels were observed. Unfortunately, no workers of the stand with the bubble mat participated in this study.

We used two sets of antibody measurements: a quantitative ELISA, and a micro-agglutination test. The ELISA measures antibody levels on a continuous scale, while the micro-agglutination test only gives results in terms of 2-fold dilution steps. As we observed before that exposure seems to cause a moderate increase in antibody level, the ELISA had higher sensitivity in this epidemiological study. However, the micro-agglutination test results showed that it was *L. pneumophila* serogroup 1 rather than serogroup 6 which was being spread in hall 3, although both serogroup 1 and serogroup 6 strains had been cultured from the whirlpool in hall 3.

7.3 Comparisons with findings in the literature

We studied only those exhibitors who were exposed to L. pneumophila, but who did not acquire LD themselves.
Some other studies of antibody titers among exposed persons were carried out in connection with outbreaks of LD.
In 1985 an outbreak occurred in patients who had visited the outpatient clinic of a hospital in Stafford (UK) in April. The attack rate in these patients was 0.6%, while that in persons accompanying these patients (presumably healthy) was 0.2%. No cases were observed in the hospital staff.[19]. These attack rates seem somewhat lower than those in the outbreak studied here, in which the overall attack rate of LD in visitors to the exhibition was 0.2%, but where the daily attack-rate increased from 0.1% on the 23rd of February to 0.5% on the 27th of February, and the attack rates in the population of exhibitors were 0.6% overall, 2.8% in hall 3 (0.2% in other places) and 4.3% within 15 meters of the whirlpoolspa.
Investigation of the Stafford outbreak included determination of antibodies to Legionella by immunofluorescent antibody assay (IFA) in a small group of obstetric patients who had visited the outpatient clinic in the second trimester of their pregnancy, and in hospital staff. IFA titers ≥ 16 were observed in 10% of obstetric patients (compared to 3% in a community sample of mixed gender and older age). The prevalence of IFA antibodies ≥ 16 was 30% in the entire hospital staff, in contrast to 3% in staff of two other hospitals who did never visit the infected hospital. Prevalence was 46% in staff working in areas served by the same air conditioning plant that served the outpatient clinic, and 23% in those working area's served by other plants.
It is noteworthy that in this study an antibody response was also observed in nurses on wards where none of the vulnerable patients acquired LD, probably because they were exposed to doses too small to cause LD. This is similar to the findings in our study, where increased

titers as compared to a reference population were also observed in those who indicated that they had not been in hall 3.
In the Stafford outbreak, influenza-like complaints were also more common in staff working in areas served by the contaminated air conditioning plant, but the magnitude of this effect is not reported. Two symptoms were found to be independently associated with the presence of Legionella antibodies: a dry cough and not having a sore throat. Especially more antibodies were found in the group with both a dry cough and no sore throat. In our study no relation was seen between a dry cough and antibody titers, although we did find a relation between having worked in hall 3 and having a dry cough.

In 1993 a small outbreak of LD (4 cases) occurred in Sydney, Australia[3]. Two cases had visited a seminar for retired persons held in a hotel, the other two had been in the vicinity of this hotel. All registered attendants (n=184) of the seminar were invited to participate in this study. Thus the attack rate in seminar attendants is 1.1%, i.e. within the range of the attack rates observed in our study. Indirect fluorescent antibody (IFA) testing was used for *L pneumophila* serogroups 1-6, both in these visitor and in blood sample from a community study. 22% of seminar attendants had IFA titers ≥ 128, against 4% of the community sample. Symptoms compatible with Legionellosis were seen in 27% of those with IFA titers ≥ 128, against 16% of those with titers <128. This difference, however, is not statistically significant. The symptom that stood out most was fever (24% against 6%, p=0.005).

A group of Legionnaires attending the convention in Philadelphia (the first discovered outbreak of LD), but who were not designated as having had LD were followed, and antibodies were determined in blood samples taken two years later[15]. A control groups of legionnaires not attending the conventions was used. In the Philadelphia outbreak, the attack rate was 9.0% in those residing in the convention hotel, and 4.0% in all persons present at the convention[11].
IFA titers against Legionella serogroup 1 were determined by IFA, both IgM and IgG.
The prevalence of IgG antibodies titer ≥ 1:64 was 28% in those who attended the convention, and 0% in those who did not; the prevalence of IgM antibodies ≥ 1:32 was 27% and 0% respectively. However, 25% of those who attended the convention had had illness within 2 weeks after the epidemic, and thus might have had a mild form of LD. It was noted that shortly after the time of the epidemic 29% of personnel working in the hotel lobby (one of the places where the exposure to *L. pneumophila* was probably high) had IFA titers of 1:64, considerably higher than that in persons not associated with the outbreak. This also confirms our finding that antibody levels tend to increase only moderately in those who are exposed but do not acquire LD.

All the above-cited studies have used antibody titers to show that an exposure had occurred in a certain building or among a certain group of persons. However, to our knowledge, this is the

first study that showed a spatial pattern of antibody titers thus implicating one area of a large hall.

7.4 Answers on research questions

7.4.1 What was the source of the epidemic?

It is clear from our results that the main LD spreading source should be sought in hall 3. The exact location, however, can not be inferred from our results with any certainty. From the two potential sources in hall 3 (the whirlpool and the bubblemat) our data seem to indicate the whirlpool (table 10) slightly stronger, but this is far from conclusive. However, in the other parts of the study a simple risk assessment was carried out of all products capable of spreading *Legionella* spp, based on interviews of exhibitors on the use of products (see section 2.1). It showed that a whirlpool in hall 3 carried more risk than the bubble-mats in hall 3, due to an more optimal growing temperature of the water and a much larger aerosolizing capacity. In the environmental investigation *L. pneumophila* was grown only from water and swab specimen collected from the whirlpool in hall 3 (growth in 14 specimen), the whirlpool in hall 4 (growth in 1 specimen) and a sprinkler installation in hall 8 (1 specimen). Primary culture plates of samples of the whirlpool in hall 3 grew considerably more colonies as compared to plates of the whirlpool in hall 4 and the sprinkling installation in hall 8. Genotyping of the environmental isolates (PFGE and AFLP) gave three different genotypes: two among serogroup O1-isolates and one among serogroup O6-isolate. One of the two genotypes among O1-isolates was identical to that of 28 of 29 available clinical isolates of LD-patients, the other was identical to the remaining clinical isolate. The dominant O1-genotype was cultured from both whirlpools, the other O-1 and the O-6 were cultured from the whirlpool in hall 3 and the sprinkling installation in hall 8 [6].

7.4.2 Attack rates

In chapter 6 the attack rates are given for Legionnaire's Disease (LD), being a serological case of infection with *Legionella pneumophila* (without having LD) and of Legionellosis resembling health complaints (without having LD).
The attack rates of LD in exhibitors not working in hall 3 is similar to the attack rate in visitors, while that of exhibitors in hall 3 is more than 10 times as large.
The attack rate for probable serological cases (defined both based on IgG and IgM) is 26% in hall 3 and less than 10% in the other halls, against 6% in the serumbank population.

A Bayesian estimation of the infection rate in hall 3 and 4 shows a very high rate of infection in hall 3 (80%) but also a reasonably high rate in hall 4 (25%). Infection here is defined as having a higher antibody level than expected based on the antibody levels in the serum bank sample.
Although those working in hall 3 reported symptoms more often than those working elsewhere, no statistically significant differences in legionellosis resembling health complaints (without having LD) were observed between both groups (in 25.2 % of those in hall 3 and 21.6% of those working elsewhere). Individual symptoms reported more often in hall 3 were a dry cough, listlessness or apathy, stomachache and earache.

7.4.3 Is there any evidence of Pontiac fever occurring in this group?

Pontiac fever is diagnosed as influenza like symptoms combined with an increase in antibodies against Legionella. We see no evidence of such a form of Pontiac fever occurring in this group, as there are only a few persons who have both a high antibody titer and also had complaints during or just after the Flora. As Pontiac fever has a very high attack rate, it is unlikely that an outbreak of Pontiac fever would have been limited to so few persons. It is more likely that these persons had a mild form of LD or that they suffered an unrelated influenza-like illness.

8. Conclusion

This study clearly shows that the main LD spreading source should be sought in hall 3. From the general knowledge on optimal growing conditions of *L. pneumophila* and on aerosol-spreading potential of devices, the whirlpoolspa is by far the most likely source of *L. pneumophila* containing aerosol in this hall. This is confirmed by the environmental investigation, in which this whirlpool was the only device in hall 3 found to be contaminated with *L. pneumophila*, while it was also more contaminated than contaminated devices found in other halls. Moreover, both genotypes of *L. pneumophila* cultured from LD-patients were grown from samples from this whirlpool. In the case-control study[5], the whirlpool in hall 3 was also implicated as the likely source. Therefore it can be concluded that aerosols from the whirlpool in hall 3 are the main source of this epidemic of LD.

In those who are exposed to this aerosol, but who do not develop LD, such an exposure seems to cause a slight elevation of antibody titers, but we could not demonstrate that such an elevation is accompanied by a substantial amount of milder forms of disease such as Pontiac fever.

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Appendix 1: Mailing list

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5. Inspectie voor Waren en Veterinaire Zaken, drs. D.H. Meijer
6. Directie Gezondheidsbeleid, VWS, Drs. A.A.W. Kalis
7. Directie Voorlichting en Communicatie, VWS, Drs R.A.C. Praat
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9. Landelijke Coördinatiestructuur Infectieziektenbestrijding
10. Officier van Justitie Alkmaar, mr. C.P.A.C. van Riel
11. Gemeente Stede Broec
12. Westfries Gasthuis
13. Mr. L.F. Keyser-Ringnalda, Officier van Justitie, Alkmaar
14. Politie Noord-Holland Noord, J. Oost
15. Politie Noord-Holland Noord, B. Pannekoek
16. Voorzitter van de Gezondheidsraad.
17. Artsen infectieziektenbestrijding GGD’s
18. Artsen-microbiologen van de streeklaboratoria
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35. Stichting Westfriese Flora
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57. Auteurs
58. Hoofd Voorlichting en Public Relations RIVM
59. Bibliotheek RIVM
60. Bureau Rapportenregistratie
61. Reserve

Appendix 2: Description non-response

A non-response form was returned by 180 persons, indicating a reason for non-response. These are listed in table 27. In table 28 the reasons for non-response are given by main place of work.

Table 27: Reasons for non-response as given on the non-response form

Reason non-response	Number	Percentage
Presence on Flora too short	19	11
Receive package too late to participate*	21	12
Has not been ill	37	21
Did not visit the consumer-products fair	15	8
Did not visit the Flora at all	38	21
Has problems with blood puncture	9	5
No time	10	6
No reason given	10	6
Not interested	3	2
Due to illness	1	1
Has been already tested for Legionnaires' disease	11	6
Got a double package	3	2
Has faith in own immunity system	1	1
Participates already as a control in case-control study	2	2
Total	180	100

* Partly these are persons that where out of town at the moment of the study; partly these are persons who feel that the study started too late

Table 28: Reasons for non-response by main place of work (only for those who gave a main place of work)

	Worked at consumer fair (n=54)		Worked elsewhere (n=67)	
Reason for non-response	Number	Percentage	Number	Percentage
Presence on Flora too short	5	9	10	15
Receive package too late to participate*	8	15	11	16
Has not been ill	15	28	21	31
Did not visit the consumer-products fair	0	0	9	13
Did not visit the Flora at all	0	0	1*	0
Has problems with blood puncture	5	9	3	4
No time	5	9	4	6
No reason given	5	9	3	4
Not interested	2	4	1	1
Has been already tested for Legionnaires' disease	7	13	3	4
Has faith in own immunity system	1	2	0	0
Participates already as a control in case-control study	1	2	1	1

* Worked outside

8 of the 11 persons who had been tested for Legionnaires' Disease answered that they had been ill after February 15th, 2 answered that they had not been ill, and 1 did not answer the question. 2 of the 8 persons who had been ill, however, had fallen ill before or on the opening day of the Flora, exposure therefore being unlikely. One other person had fallen ill 16 days after the end of the show.

6 other non-respondents indicated that they had been ill: 1 before the opening of the Flora, and 1 more than 20 days after the end of the Flora. Of the other 4, 2 had worked on the consumers-products fair, and 2 had worked elsewhere. Of all the 11 persons who had fallen ill between February, 21st and March, 20th, 7 had worked at the consumer-products fair and 4 elsewhere.

The non-response survey therefore indicates that non-responders working on the consumer fair had been ill more often, but when falling ill, had also been tested more often for Legionnaires' Disease. No subject indicated that evidence for Legionnaires' Disease had been found.

Appendix 3: Incidence of Subclinical Infections with *Legionella Pneumonia* at an Outbreak in The Netherlands:An analysis of Mixtures Using Data Augmentation.

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Summary

Infections with Legionella bacteria can cause a potentially lethal form of pneumonia (legionnaires' disease). The bacteria grow in lukewarm water and can cause infection in man when aspirated or inhaled in the form of aerosols. In 1999 a major outbreak, causing 29 deaths, occurred among visitors and exhibitors of a consumer fair in The Netherlands. The epidemiology of subclinical infections is largely unknown, as there is no reliable method to diagnose such infections. In order to help identify the source of the infection and to explore the commonness of subclinical infections IgG and IgM antibody levels among exhibitors were compared to those among a representative sample of the Dutch population. Exhibitors were assumed to comprise both infected and uninfected individuals. Thus, their antibody levels were modelled as a mixture distribution. As infected individuals are expected to cluster around a point source, the spatial aspect of the spread of infections was taken into account. To estimate the distribution of antibody levels among infected individuals and to impute infection status among exhibitors, data augmentation was used. Subclinical infection appeared to be very common and its frequency declined with the distance from a whirlpool, the putative source of the outbreak.
Key Words: *Legionella* spp, data augmentation, mixture analysis, Bayesian methods, spatial statistics.

Introduction

Legionnaires' disease is a serious pneumonia which can be life-threatening if not timely treated with appropriate antibiotics. It is caused by *Legionella* spp, which grow in stagnant water at temperatures between 25-45 ^{o}C. The route of infection is by aspiration or inhalation of aerosols. The disease was first recognised in a large outbreak among U.S. veterans in Philadelphia in 1974, which gave the disease its name[1]. Since then many outbreaks have been identified, both in the community as well as nosocomial[2]. Many isolated cases are probably never correctly diagnosed. Currently, *Legionella* is believed to be the causative organism in 1-5% of cases of community-acquired pneumonia.[3]

Many aspects of the epidemiology of the infection are still unknown. For example, evidence from outbreaks suggests that among those exposed only a small minority will get overt disease; however it is unknown whether this is because few exposed people actually get infected or whether many infections are asymptomatic or subclinical.

In the second week of March 1999, an exceptionally large number of patients with pneumonia were admitted to the Westfriese Gasthuis-hospital in Hoorn, the Netherlands. An exploratory case-control study indicated that case-patients were much more likely to have visited the "Westfriese Flora" than their neighbourhood controls. The "Westfriese Flora" is an annual flower exhibition combined with an agricultural and consumerproducts fair at the village of Bovenkarspel. In 1999 it was held from February 19 till 28.

This led to the discovery of the largest outbreak of Legionnaire's Disease (LD) in the Netherlands so far, with 132 confirmed, 53 probable and 58 possible cases, of whom 29 died. Details of the epidemic will be published elsewhere[4].

The National Institute of Public Health and the Environment (RIVM) was contacted to identify and investigate the source of the outbreak. It carried out four independent separate sub-studies:

1. **Monitoring**. Keeping a national case-register to monitor the epidemic.
2. **Environmental study**. Collection and analysis of water samples and environmental swabs of all water-using products that had been on display at the exhibition, and which were still available. Culturable *Legionella* strains were subjected to DNA fingerprinting and compared to DNA fingerprints of such strains cultured from patients.
3. **Case-control study**. A case-control study in which confirmed and probable cases from the case-register were interviewed about their visits, and compared to disease-free visitors.
4. **Serology**. All firms who had registered as exhibitors on the consumer-products and agricultural fair were contacted and requested to supply names and addresses of all persons who had worked at their stand. Thus, approximately one month after the end of the exhibition, a questionnaire and receptacles for urine and blood collection (the latter to be done by a physician or nurse) were sent to 1616 persons without overt LD, of whom 844 responded. The questionnaire asked about health status before and after the exhibition

and whereabouts during the exhibition. Blood-samples were tested for IgG and IgM antibodies against *L. pneumophila* with quantitative indirect ELISA [5]. For comparison purposes, 481 randomly selected blood samples from a nationally representative serumbank[6] (of 9948 sera) were tested for the same antibodies at the same laboratory using identical methods and reference materials.

Here we report on the serology study. The availability of antibodies in non-diseased but probably exposed individuals provided us with the opportunity to address the issue of the occurrence of asymptomatic or subclinical infections. A cluster of infected persons would suggest a source. In order to address the dual issue of source identification and asymptomatic infections we attempted to determine who amongst the exhibitors had experienced an infection.

Methods

A problem with classification of individuals as infected or uninfected was the absence of adequately validated cut-off points for antibody titres for diagnosing recent or active infection with *Legionella*. This is due to the absence of studies using these tests in samples from subjects with demonstrated subclinical infection. Even the number of studies with established clinical subjects are limited[7].We therefore proceeded as follows.
As the highest antibody levels were observed in those working at the consumer-products fair (and not at the flower exhibition or the agricultural fair), we limited the analysis to this group (n=382). For details of the sample we refer to the main part of this report. The consumer products fair comprised two halls (hall 3 and hall 4), separated by a wall with two passages. The size of the two halls combined was approximately 65 by 95 meters.
For analysis exhibitors were considered to consist of two subgroups: those (having been) infected with *L. pneumophila* and those uninfected. Those uninfected were expected to have the same distribution of antibody titres as the serumbank samples.

As the logarithms of antibody levels in the serumbank closely resembled a normal distribution, we assumed these log values to follow a normal distribution.
Thus the distribution f_{exh} among the exhibitors of log antibody titres was assumed to be the following mixture distribution:

$$f_{exh} = \lambda f_i + (1-\lambda) f_u$$

where $f_i = N(\mu_i,\sigma_i^2)$ and $f_u = N(\mu_u,\sigma_u^2)$ denote the distributions of the log(titre) in infected and uninfected respectively and λ denotes the mixing proportion. The parameters μ_u and σ^2 could be estimated from the serumbank sample and were treated as known. Thus the only unknown parameters were λ and μ_i .

In order to avoid separate analyses for log(IgG) and log(IgM), and to have more "discriminating power", we decided to use a linear combination TS (titre score) of the two. To identify the "best" linear combination of the two logtitres, i.e. the direction in which the probability of being infected increases most rapidly, logistic discriminant analysis was applied to the two groups of blood samples (from the serumbank and the exhibitors respectively).
This yielded TS = 0.30 log(IgG) + 0.50 log(IgM) as the "best" discriminating function. An almost identical linear combination was obtained when instead of the logit link, either the probit or the complementary log-log link was used. As expected from a sum of two approximately normally distributed variates the distribution of TS appeared to be well approximated by the normal (Figure 10).
The mean of TS in the serum bank sample was 2.036 with a standard deviation of 0.544; in the exhibitors the mean was 2.373 with a standard deviation of 0.631. As the variance among the exhibitors was only slightly larger than that in the serum bank sample, we further simplified the model by assuming $\sigma_i = \sigma_u = \sigma$.
Thus antibody levels among uninfected exhibitors were considered to have the same distribution as those in the serumbank sample and the distribution of the infected was assumed to have the same distributional shape, but shifted to the right. Consequently, the extra variance among the exhibitors compared to the serumbank was assumed to be entirely due to the effect of mixing infected and uninfected populations. The expected value of TS among the infected, μ_i, was of course unknown and had to be estimated from the data.
As TS did not appear to depend on age and gender, we ignored these factors in our analysis.

Thus, a training set from one - the uninfected - of the two populations constituting the mixture is available, but not from the other – infected - population. Methods to analyse this type of mixture problem have been developed[8-13]. However, these methods ignore a key feature of our data, viz. its spatial structure: one can expect infected cases to cluster around the source. We felt that this feature would be informative and therefore needed to be taken into account. As the missing-data aspect (of infection statuses) is a key aspect of these data, we resorted to data-augmentation methods for analysis[14]. In order to make effective use of these methods we made the additional assumption that the distribution of an individual's value of TS depends **only** on his infection status. Thus, conditional on an individual's infection status, the distribution of TS does not depend on other variables, such as the location of his place of work. We call this the *conditional independence* assumption. The converse however, was **not** assumed to hold true. In fact, the probability of being infected given TS was assumed to depend upon an individual's prior probability of being infected, which was supposed to depend on the prevalence of infection among the individuals working in his vicinity.

Data augmentation

The objectives of the analysis are to make inferences about the unknown parameter μ_1 and to impute the infection status of each exhibitor.
We used data augmentation as follows: iteratively we took two steps, one to impute the infection status for the "current" value of μ_i and one to update μ_i given imputed infection statuses; and continued until convergence (i.e. the process has become stationary) had been reached:

1. This step (step 1) is called[14] the *imputation step*. Given the current value of μ_i, m values $\mathbf{I}^1$, $\mathbf{I}^2$, .., $\mathbf{I}^m$ of the vector $\mathbf{I} = (I_1, I_2, .. ,I_N)$ are generated, where I_j is the infection status (0=uninfected, 1=infected) of individual j. Thus I^k_j is the generated infection status of individual j of the k-th (out of m) vector. For m we took a value of 20.
2. This step (step 2) is called the *posterior step*. The posterior of μ_i given $\{\mathbf{I}^k\}$ and $\mathbf{TS}=(TS_1,TS_2, .., TS_N)$ is calculated. Then a new value of μ_i is obtained by sampling from this posterior distribution.

As convergence of the process appeared to be rapid, we calculated a total of 150 iterations, of which the first 50 were treated as a "burn- in" and therefore discarded.
Below we will describe each step in detail.

Step 1. Step 1 is in itself an iteration process (each step over all N employees of the hall). This worked as follows.
For each individual (here we use number 1 as an example) we needed to calculate $Pr(I_1 \mid (TS_1, ..,TS_N), \mu_i)$. As $Pr(I_1 \mid (TS_1, ..,TS_N), \mu_i)$ could not be estimated directly, we **sampled** instead from the distribution $Pr(I_1 \mid I_2, ..,I_N, (TS_1, ..,TS_N), \mu_i)$, given (equilibrium) realisations of $I_2, ..,I_N$. As,

$Pr(I_1 \mid (TS_1, ..,TS_N), \mu_i) = Pr(I_1 \mid I_2, ..,I_N, (TS_1, ..,TS_N), \mu_i). Pr(I_2, ..,I_N \mid (TS_1, ..,TS_N), \mu_i)$

Drawings of $I_2, ..,I_N$ followed by drawings from the conditional (0,1) distribution $Pr(I_1 \mid I_2, ..,I_N, (TS_1, ..,TS_N), \mu_i)$ are essentially realisations of draws from $Pr(I_1 \mid (TS_1, ..,TS_N), \mu_i)$.

We have (using the conditional independence assumption above and Bayes' theorem) that (for inf = 0,1),

$$Pr(I_1 = inf \mid I_2, ..,I_N, TS_1, (TS_2, ..,TS_N), \mu_i) =$$
$$Pr(y_1|I_1=inf, I_2, ..,I_N, \mu_i)Pr(I_1=inf|I_2,\ldots,I_N, \mu_i)/$$
$$\{Pr(TS_1|I_1=0, I_2, .,I_N, \mu_i)Pr(I_1=0|I_2,.,I_N, \mu_i) +$$
$$Pr(TS_1|I_1=1, I_2, .,I_N, \mu_i)Pr(I_1=1|I_2,.,I_N, \mu_i)\}$$
$$=$$
$$= P(TS_1|I_1= inf, \mu_i)Pr(I_1= inf|I_2,\ldots,I_N, \mu_i)/$$

$$P(TS_1|I_1=0, \mu_i)Pr(I_1=0|I_2,\ldots,I_N, \mu_i) + P(TS_1|I_1=1, \mu_i)Pr(I_1=1|I_2,\ldots,I_N, \mu_i) \}$$

As the purpose of $Pr(I_1|I_2,\ldots,I_N, \mu_i)$ is to express the "prior" probability of an individual's infection status provided by knowing the infection status of all other exhibitors, we assumed it not to depend on μ_i explicitly. We thus needed to calculate $Pr(I_1|I_2,\ldots,I_N)$. The functional form of this probability should reflect the idea that - if it is known that several exhibitors working nearby an individual have been infected - the "prior" probability for that individual that he is also infected is increased. Several "models " for $Pr(I_1|I_2,\ldots,I_N)$ that reflect this idea are possible. The simplest is probably to take a neighbourhood H (for instance a circle with a radius of ρ (e.g. 10) meters). Then, within that neighbourhood, count: a) the *current* estimated number of infected individuals M_{inf} (except the individual of interest, here indexed with 1), and divide this by b) M_H the total number of individuals (also except the individual of interest) working within that neighbourhood.
Thus,

$$Pr(I_1=1|I_2,\ldots,I_N, \mu_i) = M_{inf}/ M_H$$

Instead of such a rectangular kernel, we used the tricube kernel[15] with a (rather arbitrarily chosen) radius ρ of 10 meters, thus

$$M_{inf} = \Sigma_{i\neq 1}\, \omega_i\, I_i \;; \quad M_H = \Sigma_{i\neq 1}\, \omega_i$$

and

$$\omega_i = (1-(d_{1,i}/\rho)^3)^3 \; (d_{1,i} <= \rho); \qquad \omega_i = 0 \; (d_{1,i} > \rho);$$

where $d_{1,i}$ denotes the Euclidean distance (in metres) between the workplace of exhibitor 1 and exhibitor i.

To avoid "prior" probabilities of 0 or 1 we used instead

$$Pr(I_1=1|I_2,\ldots,I_N, \mu_i) = (M_{inf} +0.5)/ (M_H +1)$$

The distance between individuals in two different halls was calculated as the shortest distance via either of the two passages between them.

This process was iterated (for given μ_i) until convergence was reached. Then another m samples were taken, and retained for step 2. This completed (a single) step 1.

Step 2. The likelihood of μ_i given $\mathbf{I}^k$ and **TS** is,

$$Pr\,(\mathbf{TS}|\mathbf{I}^k,\mu_i) = (\sigma^{-N}(2\pi)^{-N/2})\Pi\, \exp(-0.5(TS_i - \mu_u)^2/\sigma^2)\; \Pi\, \exp(-0.5(TS_i - \mu_i)^2/\sigma^2)$$

Where the first product is over the $I_j^k=0$ and the second over the $I_j^k=1$. Only the second product involves the unknown parameter (μ_i). Thus the first product is a "constant" factor.

Step 2 involves drawing from the posterior distribution of μ_i. We assumed a flat prior so that the posterior is proportional to the likelihood.
For each of the m realisations (samples, draws) $\mathbf{I}^1$, .., $\mathbf{I}^m$ from the equilibrium distribution the above constant factor was calculated as weight w_k. Then one (j, say) of the numbers 1, 2,…,k was randomly drawn with probability proportional to the weights w_k.
Given number j, a new value of μ_i was drawn from the normal distribution $N(TS^j,\sigma^2/N_j)$, where TS^j is the mean of the N_j observations (the "**TS**'s") that have $I_k=1$ in the j-th draw selected above. This completed (a single) step 2.

To explore sensitivity of the results of the use of the serumbank samples, we repeated all analyses with serum taken from exhibitors working at the agricultural fair, which was situated at the other end of the "Westfriese Flora".
The above analyses were programmed in ANSI-C (available from the authors on request) and run on a standard PC.

Results

μ_i was estimated to be 2.468 with a standard error of 0.215. Some 80% and 25% of exhibitors in hall 3 and hall 4 respectively were estimated to have experienced subclinical infection. Figure 12 displays the mean estimated probability of infection for exhibitors per stand in the consumer products fair. Clearly hall 3 contains the source. Results using the exhibitors from the agricultural fair as non-exposed sample were very similar (results not shown). Table 29 gives the estimated prevalence of infection, both by hall, and by distance to the putative source (see discussion).

Discussion

The most likely aerosol spreading devices on the consumer fair were two whirlpool spa's in hall 3 and hall 4 (see figure 11-13). The evidence from our analyses point to hall 3. This is in agreement with the clinical data, from which we estimated an attack rate of 2.8% of confirmed and probable Legionnaires' Disease in exhibitors in hall 3, and of 0.4% in hall 4, as well as with the microbiological results, in which primary culture plates of samples from the whirlpool in hall 3 grew considerably more colonies as compared to plates from the whirlpool in hall 4. DNA fingerprinting of the cultured isolates gave three different genotypes: two serogroup O1-isolates and one serogroup O6-isolate. One of the O1-isolates

was identical to 28 of 29 available clinical isolates of case-patients; the other O1 isolate was identical to the remaining clinical isolate. The dominant O1-genotype was cultured from both whirlpools; the other O1 was cultured from the whirlpool in hall 3 and a sprinkling installation at the agricultural fair [8]. This also points to the whirlpool in hall 3 as a more likely source. The estimated probability of infection was particularly high near the whirlpool in hall 3, although the pattern is not centred exactly at this place.

We estimate that on average 24.8% of those in hall 4 and 80.0% of those in hall 3 were infected with *L. pneumophila.* This high level of asymptomatic infections is surprising. With attack rates of clinical *Legionella* disease of 2.8% and 0.4%, among exhibitors in hall 3 and hall 4 respectively, most infections with *Legionella* or at least with the type isolated here are apparently asymptomatic. This may explain, if true, the widespread presence of measurable amounts of IgG and IgM antibodies among the serumbank sample, although cross-reactivity with other antigenically related micro-organism may play a role.
We have made several assumptions to arrive at our conclusion. One of these is the identity of the variances of the response variable TS in uninfected and infected. It may be possible that the variance among the infected is larger than among the uninfected (the converse seems to be less plausible). If so, the prevalence of infection should be even higher than we estimated. However, if the prevalence of infection would have been near 100%, it is unlikely that we would have seen a pattern of infection within the halls as observed.
Another assumption we have made is that the distribution of TS conditional on the infection status does not depend on the position of the workplace within the hall. This would not be true if there were e.g. a substantial dose-response effect, with exhibitors working near the source developing higher levels of antibodies as a result of more prolonged and intense exposure to the pathogens. There is no way to empirically verify this assumption, as we have no objective diagnostic criterion to unequivocally establish infection.

Another critical assumption is that the serumbank samples can be used as “controls”. The serumbank samples had been stored for about 4 years at –80 ^{o}C. Since antibody levels remain stable under these conditions, this probably hardly affected our results[16]. However, there are several reasons why the distribution of antibody titres among the uninfected exhibitors may not be identical to the distribution in the serumbank population. First, the fair was held a few weeks after an influenza epidemic, which might have interfered with immune responses or given rise to unexpected cross-reactivity and thereby increased the antibody levels in the population.
Second, although the serumbank was constructed to represent the general population, the exhibitors are likely not fully representative of the general population in every sense, e.g. they may have been more frequently exposed to *Legionella* as a consequence of their work.
Thirdly, the persons performing the immunological assays were not blinded with respect to whether the samples came from the serumbank or from the exhibitors (although they were blinded with respect to the place of work of exhibitors). However, in view of the nature of the

test, it is not very likely that this will have biased the results. Although there are many potential sources of bias, we nevertheless feel that these cannot have influenced the distributional pattern within and between the halls as using exhibitors from the agricultural fair as a training set (instead of the serum bank) yielded a similar spatial pattern.

We used data augmentation as - to our knowledge - it is the only method that could take into account our assumptions about the spatial distribution of the infections. A drawback of such a simulation method, however, is that repeated analyses never yield identical results. Its computationally intensive nature is no longer a problem on today's PC's.

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Table 29: Average probability of infection as estimated when using the serum bank titers (n=481) as the titer distribution of unexposed, and when using the exhibitors at the agricultural fair (n=276) as such.

	With serumbank as reference	With exhibitors agricultural fair as reference
Work place:		
Hall 3 (n=140)	80.0%	70.0%
Hall 4 (n=242)	24.8%	19.8%
Distance to the whirlpool in hall 3		
< 15 meter (n=57)	85.2%	80.8%
15-30 meter (n=22)	78.7%	66.5%
30-60 meter (n=146)	44.5%	35.5 %
60-90 meter (n=157)	25.9%	21.1%

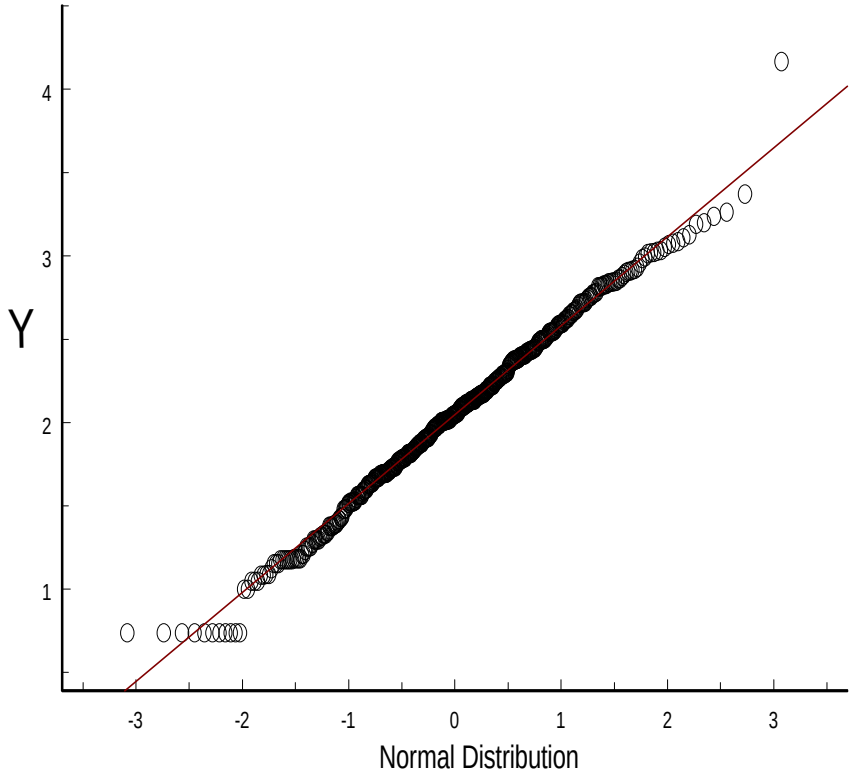

Figure 10: q-q plot of TS in the serum bank sample (n=481)

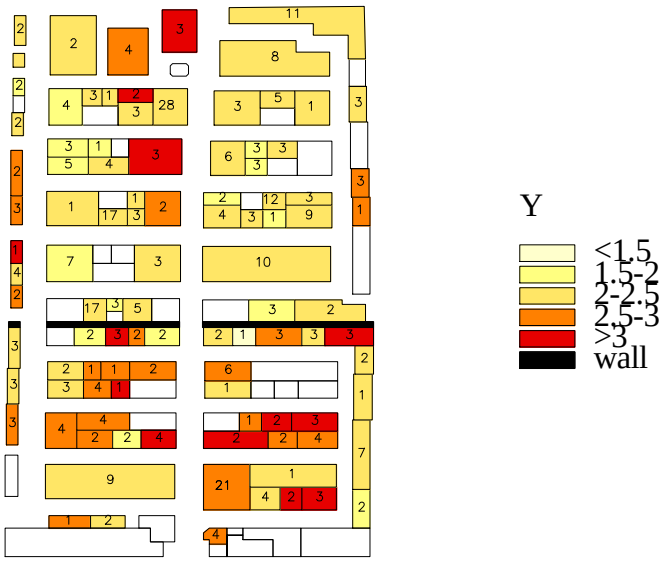

Figure 11: Mean value of TS per stand in the consumer products fair. The part above the wall is Hall 4, the part below the wall is Hall 3.

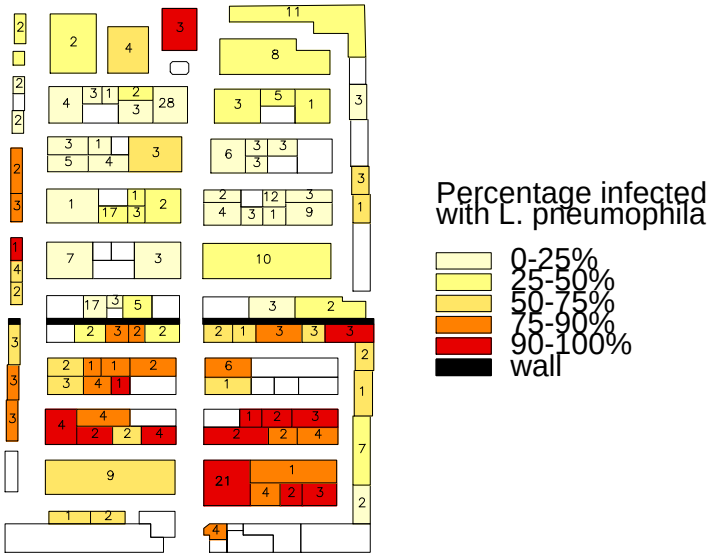

Figure 12: Estimated prevalence of infection per stand. The part above the wall is Hall 4, the part below the wall is Hall 3. The number of subjects included in the analysis is printed in each stand.

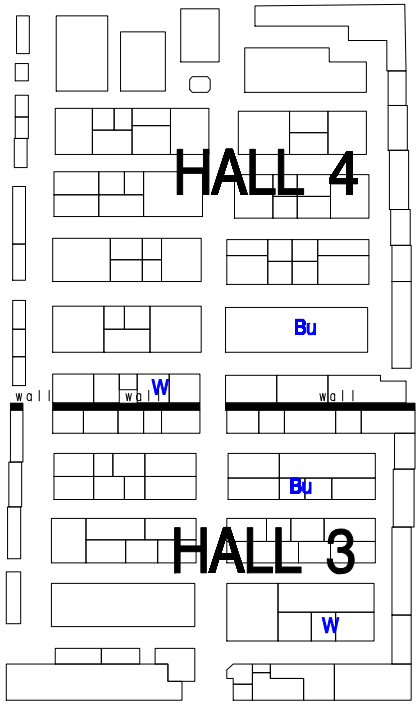

Figure 13: Lay-out of Hall 3 and 4. The whirlpools are denoted by W, the baths with a bubblemat by Bu

Appendix 4: Extra table

Table 29: Absolute values of geometric mean antibody titers against L. pneumophila, all exhibitors (also those only present before February, 23rd).

Group	IgG-Elisa in U/ml		IgM-Elisa in U/ml		IgM type 1 1: 32 or more	IgM type 6 1:64 or more
	First sample (n=844)	Maximum Paired samples (n=714)	First sample (n=844)	Maximum paired samples (n=714)	First sample (n=761)	First sample (n=761)
Hall 3	28.6	33.9	24.1	25.2	6.7	2.0
Hall 4	18.9	20.4	14.2	14.5	1.6	1.2
Agricultural fair	21.0	23.7	15.4	16.8	1.3	1.8
Flower show	17.9	21.6	15.2	17.4	0.0	3.1
Other places	20.7	19.1	14.7	16.0	0.0	2.3
Several halls	17.6	20.7	17.1	17.4	2.0	2.0
All	20.7	23.5	16.5	17.3	2.4	1.7