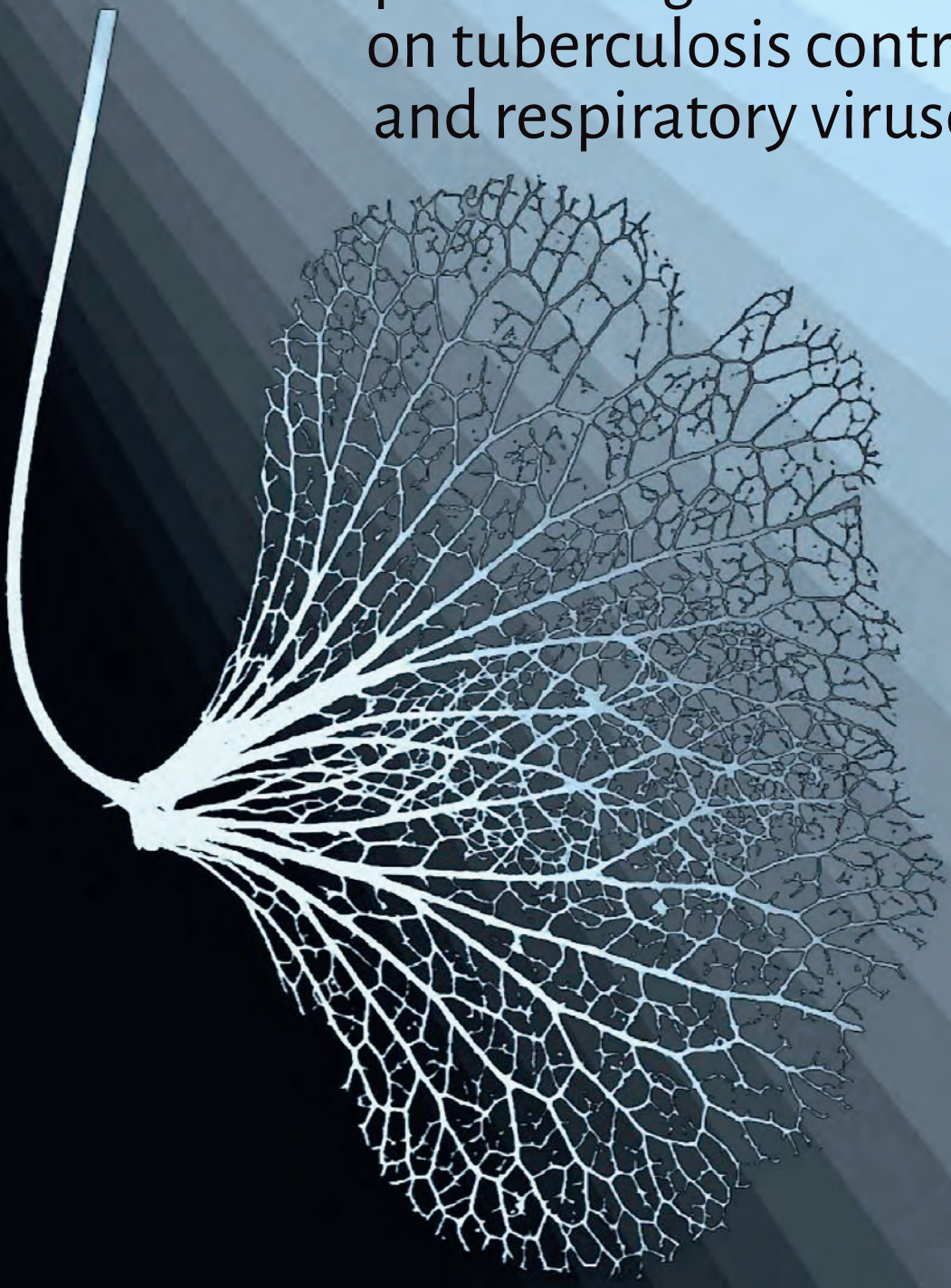
Epidemiological studies on tuberculosis control and respiratory viruses



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Thesis, University of Amsterdam (UvA), the Netherlands
ISBN: 978-90-6464-866-3
http://dare.uva.nl/dissertaties

Cover:	image by P. Nijenhuis (modified by P. Duijn)
Lay-out:	Ferdinand van Nispen, Citroenvlinder-dtp.nl, Bilthoven, the Netherlands
Printed by:	GVO drukkers & vormgevers, Ede, the Netherlands

Financial support for printing of this thesis was provided by:
KNCV Tuberculosis Foundation, AMC, Stichting tot Bevordering van de Klinische Epidemiologie Amsterdam, Delft Imaging Systems.

Epidemiological studies on tuberculosis control and respiratory viruses

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. D.C. van den Boom
ten overstaan van een door het College voor Promoties ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
op woensdag 10 juni 2015, te 10:00 uur

door Rosa Sloot
geboren te Haarlem

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Chapter 2

Clustering of tuberculosis cases based on variable-number tandem-repeat typing in relation to the population structure of *Mycobacterium tuberculosis* in the Netherlands

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Journal of Clinical Microbiology 2013;51:2427-31

Abstract

The population structure of 3,776 *Mycobacterium tuberculosis* isolates was determined using variable-number tandem-repeat (VNTR) typing. The degree of clonality was so high that a more relaxed definition of clustering cannot be applied. Among recent immigrants with non-Euro-American isolates, transmission is overestimated if based on identical VNTR patterns.

DNA typing is a powerful tool to trace tuberculosis (TB) transmission and outbreaks. Clustering of *Mycobacterium tuberculosis* isolates based on identical DNA fingerprints is commonly used as a proxy for recent transmission (1). However, this assumption is not always correct and depends on many factors, such as circulation of genetically similar strains, evolution of *M. tuberculosis* over time, transmission rate, DNA typing methods applied, duration of the study period, sampling, and effectiveness of TB control (2, 3). Various studies have shown that not all cases in DNA fingerprint clusters have epidemiological links with other cases in the cluster (4, 5). Moreover, epidemiological links have been found between cases caused by bacteria with slightly different DNA fingerprints (6). Clustering results among cases in the immigrant population especially should be interpreted with caution (7, 8), as isolates from these patients often belong to genetically compact strain lineages predominating in the countries of origin (9, 10, 11, 12).

In the Netherlands, more than 70% of all TB cases are found among foreign-born persons, and extensive information on each patient is stored in a national registry. We aimed to investigate the population structure of *M. tuberculosis* isolates among native and immigrant cases and to determine the consequences for the interpretation of recent transmission based on variable-number tandem- repeat (VNTR) typing results.

Culture-confirmed TB cases from October 2003 to December 2008 were included in this study. Patient information was obtained from the Netherlands Tuberculosis Register (NTR), held by the KNCV Tuberculosis Foundation. In total, 3,975 *M. tuberculosis* isolates were typed by IS*6110*/PGRS restriction fragment length polymorphism (RFLP) and standard 24-locus VNTR typing (13, 14) at the RIVM or by Genoscreen (Lille). Molecular data were matched with demographic data using the date of birth, sex, postal area code, and year of diagnosis, resulting in 3,793 (95%) matching cases. After exclusion of 17 foreign-born individuals because of incomplete data for several variables, 3,776 (95%) cases remained eligible.

Genotype information was uploaded to the MIRU-VNTRplus web-application (http://www.miru-vntrplus.org) (15) for phylogenetic lineage prediction, which was performed stepwise as described by Allix-Beguec et al. (16). Isolates that were part of the CAS, Beijing, EAI, *Mycobacterium bovis*, and *Mycobacterium africanum* lineages were categorized as non-Euro-American and the remaining as a Euro-American superlineage (16).

Clonal complexes, defined as groups of at least two isolates differing in not more than 3/24 loci, were identified on a minimum-spanning tree with BioNumerics software (Applied Maths, Kortrijk, Belgium), using MIRU-VNTR data and the categorical distance, which scores the number of alleles shared or different over the 24 markers used.

Multiple imputation was used to account for 184 (5%) and 37 (1%) of 3,776 cases with missing data for the variables "time since immigration at TB diagnosis" and "gender," respectively. All remaining variables were used to create five imputed data sets, and results are based on pooled statistics. Two different cluster definitions were used to investigate the interpretation of recent transmission; identical VNTR patterns and single-locus variants (SLVs). The theory behind this was that allowing SLVs to be clustered might involve genetically closely related strains that in fact share the same transmission chain. The analyses were performed separately for the Euro-American and non-Euro-American lineages and completed with SPSS 18.0 (SPSS, Chicago, IL) and statistical program R version 2.11.0.

A minimum-spanning tree was produced for all 3,776 isolates included in the analysis. In total 3,377 (89%) isolates were distributed over 83 clonal complexes (Fig. 1), whereas the remaining 399 (11%) isolates did not belong to any clonal complex. Within each complex, all the VNTR patterns represented the same lineage type, except the two largest complexes comprising 84% Haarlem strains and 67% T-specific strains.

Of the 3,776 isolates, 1,130 (30%) represented the non-Euro-American lineages (Table 1). We reasoned that recently arrived immigrant cases having nonclustered *M. tuberculosis* isolates most likely represent importation of foreign genotypes. Among the 504 nonclustered recent-immigrant cases, 239 (47%) were caused by isolates of the non-Euro-American lineages, of which EAI (45%), CAS (26%), and Beijing (18%) constituted the majority (Table 1). Cases caused by these non-Euro-American lineages originated from Asia (41%) and Africa (56%). In contrast, recent-immigrant nonclustered cases with Euro-American lineages had a higher diversity in geographical origin. Furthermore, 40 (8%) of the 504 recent-immigrant nonclustered cases originated from European countries, of which the majority (93%) were caused by the Euro-American lineages. Among the 564 native Dutch cases with nonclustered *M. tuberculosis* isolates, 459 (81%) had isolates of the Euro-American lineages, of which the majority were of the Haarlem (36%), T-specific (33%), and LAM (13%) lineages (Table 1).

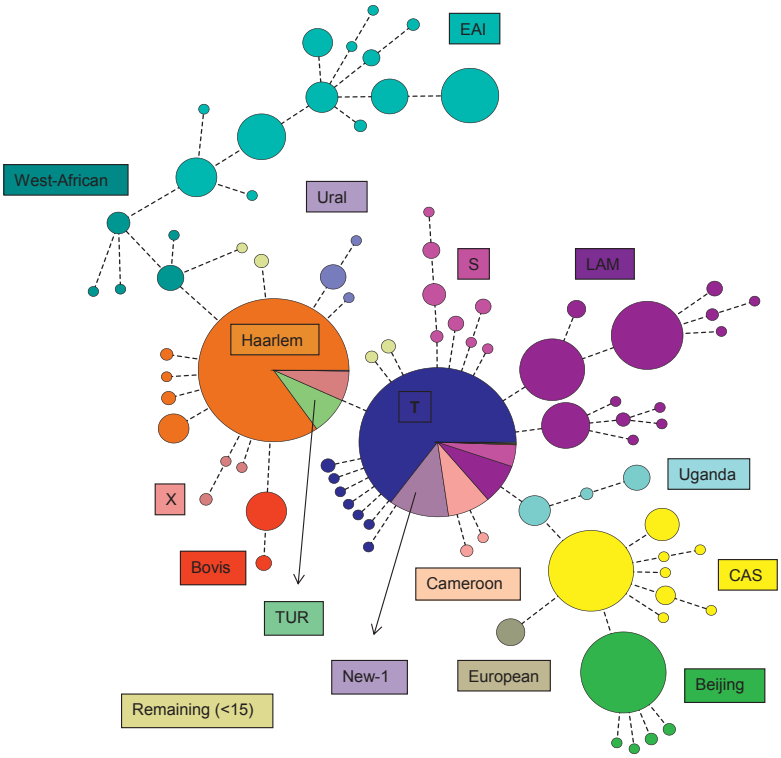

Figure 1. Identification of clonal complexes in the total study population in a minimum-spanning tree

Patient factors significantly associated with VNTR clustering in the whole study population, using identical profiles as a cluster definition, were analyzed. As observed in previous studies (17, 18), we found male sex, young age, urban residence, having pulmonary tuberculosis, and no previous treatment for tuberculosis as significant risk factors for clustering. All risk factors became less strongly associated when analyses was restricted to cases caused by non-Euro-American lineages (Table 2). Only age (<30 and 55 to 70 years) and having pulmonary tuberculosis remained significantly associated with clustering.

Table 1. Distribution of non-Euro-American and Euro-American lineages over clustered and nonclustered immigrant and native Dutch tuberculosis cases

	No. of isolates					
	Nonclustered cases			Clustered cases		
	Immigrants					
Lineage	Resident <3 yr	Resident ≥3 yr	Natives	Immigrants	Natives	Total study population
Non-Euro-American						
EAI	108	135	19	96	26	384
CAS	63	108	15	117	25	328
Beijing	42	69	37	98	28	274
Bovis	5	10	26	10	11	62
West African (I,II)	16	21	2	18	2	59
With <10 isolates	5	10	6	2	0	23
Total	239	353	105	341	92	1,130
Euro-American						
Haarlem	55	133	166	278	182	814
T specific	64	126	151	152	134	627
LAM	68	148	61	236	102	615
S	13	44	30	23	22	132
Uganda (I,II)	9	28	4	36	4	81
New-1	15	47	8	8	7	85
TUR	9	16	5	32	20	82
X	8	16	16	19	21	80
Cameroon	13	15	10	30	6	74
Ural	7	11	6	5	2	31
With <15 isolates	4	14	2	5	0	25
Total	265	598	459	824	500	2,646

Allowing SLVs to be clustered increased the clustering proportion by 24% and 57% among cases with Euro-American isolates and non-Euro-American isolates, respectively (Table 2). This resulted in a decreased magnitude of association between the risk factors and clustering, in particular for the cases caused by non-Euro-American lineages (Table 2). Similarly, the association between RFLP and VNTR clustering in cases caused by non-Euro-American lineages was reduced compared to that in cases caused by Euro-American lineages. Discrepancy between VNTR and RFLP typing (i.e., clustered by either VNTR or RFLP) was in most cases caused by VNTR clustered and RFLP nonclustered isolates (Table 2). Among 1,130 non-Euro-American isolates, 177

(16%) were clustered by VNTR and nonclustered by RFLP. In contrast, among 2,646 Euro-American isolates, 263 (10%) were clustered by VNTR and nonclustered by RFLP (Table 2).

This study shows the lineage-dependent degree of reliability of the inference on transmission. Classification of lineage type was based on the geographical association between patient origin and strain lineage, defined as Euro-American and non-Euro-American. Among nonclustered native Dutch TB cases, Euro-American lineages were most frequently isolated. Domination of these lineages among native TB cases has also been shown in other European populations (19, 20), suggesting that these lineages have been circulating in Europe for centuries (21). In contrast, recent-immigrant cases caused by nonclustered non-Euro-American strains originated from distant geographical areas.

Risk factors for recent transmission, as determined by VNTR clustering, were reduced in the non-Euro-American lineages compared to the Euro-American lineages, indicating the lineage dependence. This was further visible when testing the effect of tolerating single-locus variants in the cluster definition, as the increase in clustered non-Euro-American strains was twice as high as that among Euro-American strains, reflecting the clonality of the former strains in the study population. Furthermore, the magnitude of association between risk factors and clustering decreased after allowing single-locus variants, especially among cases with non-Euro-American isolates, thus increasing overestimation of recent transmission for cases caused by non-Euro-American lineages.

In conclusion, to remain useful in TB control practice, the definition of a cluster on the basis of VNTR typing should be a fully identical 24-locus VNTR typing result. This study further indicated limits in the interpretation of recent transmission based on clustering by VNTR typing in the recent-immigrant population. Our findings are in particular relevant for other European low-incidence countries having similar forms of immigration.

Table 2. Risk factors for clustering, with and without allowing single-locus variants to be clustered, according to lineage [a]

	Non-Euro-American lineages					Euro-American lineages					
	No locus variation		Allowing SLVs to be clustered			No locus variation		Allowing SLVs to be clustered			
Parameter	No. (%) of VNTR clustered isolates	OR (95% CI)	No. (%) of VNTR clustered isolates	OR (95% CI)	Total no. of isolates	No. (%) of VNTR clustered isolates	OR (95% CI)	No. (%) of VNTR clustered isolates	OR (95% CI)	Total no. of isolates	Total no. of isolates in study population
Sex											
Male	243 (39)	1.1 (0.9-1.4)	373 (60)	1.0 (0.8-1.3)	619	855 (54)	1.5 (1.3-1.8)	1,023 (65)	1.3 (1.1-1.6)	1,579	2,198
Female	190 (37)	1	306 (60)	1	511	469 (44)	1	616 (58)	1	1,067	1,578
Age, years											
<30	157 (40)	1.7 (1.0-2.8)	240 (61)	1.7 (1.1-2.7)	392	429 (58)	5.1 (3.9-6.7)	501 (68)	3.5 (2.7-4.6)	734	1,126
30-55	202 (38)	1.6 (0.9-2.6)	323 (61)	1.6 (1.0-2.6)	534	645 (56)	4.6 (3.5-5.9)	771 (67)	3.3 (2.6-4.2)	1,153	1,687
55-70	49 (43)	1.9 (1.1-3.4)	73 (64)	1.9 (1.1-3.3)	115	162 (46)	3.1 (2.3-4.2)	213 (61)	2.5 (1.9-3.4)	352	467
>70	25 (28)	1	43 (48)	1	89	88 (22)	1	154 (38)	1	407	496
Residence											
Urban	165 (42)	1.3 (0.9-1.6)	237 (60)	1.0 (0.8-1.3)	393	601 (58)	1.7 (1.4-1.9)	721 (69)	1.7 (1.4-2)	1,040	1,433
Rural	268 (36)	1	442 (60)	1	737	723 (45)	1	918 (57)	1	1,606	2,343
Had TB before											
Yes	12 (26)	0.5 (0.3-1.1)	25 (53)	0.7 (0.4-1.3)	47	72 (38)	0.6 (0.4-0.8)	89 (47)	0.5 (0.4-0.7)	190	237
No/unknown	421 (39)	1	654 (60)	1	1,083	1,252 (51)	1	1,550 (63)	1	2,456	3,539
Localization of TB											
PTB	228 (44)	1.8 (1.4-2.4)	332 (65)	1.5 (1.2-2.0)	514	844 (53)	1.6 (1.3-1.9)	1,026 (65)	1.5 (1.3-1.8)	1,589	2,103
EPTB	141 (31)	1	251 (54)	1	463	303 (41)	1	396 (54)	1	732	1,195
PTB+EPTB	64 (42)	1.6 (1.1-2.4)	96 (63)	1.4 (0.9-2.1)	153	177 (55)	1.7 (1.3-2.2)	217 (67)	1.7 (1.3-2.2)	325	478

Table 2 (continued). Risk factors for clustering, with and without allowing single-locus variants to be clustered, according to lineage [a]

	Non-Euro-American lineages					Euro-American lineages					
	No locus variation		Allowing SLVs to be clustered			No locus variation		Allowing SLVs to be clustered			
Parameter	No. (%) of VNTR clustered isolates	OR (95% CI)	No. (%) of VNTR clustered isolates	OR (95% CI)	Total no. of isolates	No. (%) of VNTR clustered isolates	OR (95% CI)	No. (%) of VNTR clustered isolates	OR (95% CI)	Total no. of isolates	Total no. of isolates in study population
RFLP											
Clustered	256 (74)	9.9 (7.4-13.3)	306 (89)	8.7 (6.0-12.4)	345	1,061 (84)	21.9 (17.9-26.7)	1,145 (90)	16.8 (13.5-20.8)	1,267	1,612
Nonclustered	177 (23)	1	373 (48)	1	785	263 (19)	1	494 (36)	1	1,379	2,164
Total	433		679		1,130	1,324		1,639		2,646	3,776

[a] SLV, single-locus variant (maximum threshold of one MIRU locus variation from a central strain or any other strain within the same lineage [Euro-American versus non-Euro-American lineages])

OR=odds ratio; CI=confidence interval; PTB=pulmonary tuberculosis; EPTB=extrapulmonary tuberculosis

Acknowledgements

We thank all the Municipal Health Services that reported their cluster investigations to the national surveillance unit and the microbiological laboratories in the Netherlands for their efforts to send *Mycobacterium tuberculosis* isolates to the RIVM.

P.S. is a consultant for GenoScreen, Lille, France. All other authors have declared that no competing interests exist.

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Chapter 6

Distribution and viral load of respiratory viruses differ by illness severity in adults: a comparison between community and hospital populations

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Abstract

Objective: To better understand the clinical significance of respiratory viruses, we investigated the prevalence, relative distribution and viral load of respiratory viruses in community and hospital populations by illness severity.

Methods: From 2011 to 2013, nasopharyngeal samples were collected during the influenza season from adult participants of the HELIUS study, a Dutch population-based, multi-ethnic cohort study. For comparison, routine diagnostic and demographic data were used of adult patients presenting at a hospital serving the geographical area of the cohort study. Study participants were grouped by approximated illness severity: asymptomatic and mildly symptomatic HELIUS participants and hospital outpatients, inpatients, and ICU-admitted patients. Respiratory viruses were detected by multiplex real-time PCR. Crossing point values were used to estimate viral load.

Results: Respiratory viruses were detected in 12% of the community population and 31% of the hospital population. Among virus-positive subjects, rhinovirus (RV), human coronavirus (hCoV) and human bocavirus were significantly overrepresented in the community population, while the reverse was true for influenza A virus (InfA) and human metapneumovirus. Viral load of InfA and RSV was significantly correlated with illness severity. Correlations were less clear for RV and hCoV but highest viral loads were observed in ICU-admitted patients.

Conclusion: Differences in distribution between community and hospital populations confirm differences in pathogenicity between respiratory viruses in adults. Viral load correlated with illness severity for InfA and RSV but this was less clear for viruses with reduced pathogenicity, like RV and hCoV. Determining the clinical significance of such viruses in individual hospitalized patients remains challenging.

Introduction

Rapid and accurate detection of viral pathogens facilitates early diagnosis, early identification of outbreaks, timely intervention, effective management of high-risk contacts, appropriate antimicrobial therapy, and avoidance of unnecessary laboratory testing [1]. The introduction of real-time polymerase chain reaction (RT-PCR)-based methods has greatly improved the diagnostics for respiratory viral infections. However, detection of a viral pathogen by RT-PCR does not necessarily imply causality of the illness; it is well established that subclinical respiratory viral infections do occur. Moreover, because of its high analytic sensitivity, RT-PCR may detect small amounts of viral nucleic acids, the clinical relevance of which may be difficult to interpret since, for example, these may reflect past or asymptomatic infections [2]. Multiple studies have demonstrated a correlation between viral load of respiratory viruses and severe disease [3] [4] [5] [6] [7]. Hence, quantitation of viral nucleic acids may be helpful for the clinical interpretation of a positive PCR result.

Understanding the clinical significance of respiratory viral infections is essential for improving preventive and therapeutic strategies. Most etiological studies have focused on patients presenting in health care settings with respiratory illness. However, studies in the general population could provide information on the background prevalence of respiratory viral infections and thereby contribute to our understanding of the clinical interpretation of a positive PCR result in patients with respiratory illness who are seeking health care. Therefore, the objective of our study was to compare the prevalence and relative distribution of respiratory viral infections among adult populations that vary in illness severity, ranging from asymptomatic or mildly symptomatic individuals in the general population not seeking healthcare to patients with severe disease requiring intensive care, and to evaluate whether viral load estimations could aid in the interpretation of diagnostic test results. A population-based cohort study provided the opportunity to study the prevalence, relative distribution and viral load of respiratory viral infections in the adult general population. For comparison, demographic data and routine diagnostic test results were used from adult patients who, during the same period as the cohort study, were presented or admitted to a large tertiary referral hospital which also provides non-tertiary care for the catchment area of the cohort study population.

Methods

Study population

HELIUS study

The HELIUS study (acronym for Healthy Life in an Urban Setting) is designed as a large prospective cohort study, initiated by the Academic Medical Center (AMC) and the Public Health Service of Amsterdam, to understand the unequal burden of disease across ethnic groups [8]. People aged 18-70 years, of Dutch, Turkish, Moroccan, Ghanaian, Surinamese origin were sampled from the municipal civil register of Amsterdam and invited to participate. Sampling was random but stratified by ethnicity, as defined by the country of birth of individuals or their parents as documented in the civil register [8]. Persons who were unable to give informed consent and persons who were not registered with a general practitioner were excluded. Baseline data collection started in January 2011 and is still ongoing. Data are collected through a questionnaire and physical examination, and biological samples are obtained during study visits.

During the data collection from January 2011 until June 2013, nasal and throat swabs were obtained by trained nurses or research assistants using flocked swabs (FLOQSwabs™, Copan, Brescia, Italy) and collected in a single tube containing 3 mL of viral transport medium (UTM™ medium, Copan, Brescia, Italy). The transport medium was kept at room temperature until transport to the AMC Department of Medical Microbiology on the same day. On arrival, the tubes containing the combined swabs and transport medium were vortexed, the swabs were discarded, and the medium was divided into two equal aliquots and stored at -80°C until further processing [8].

Before the physical examination at the study site, participants were asked by a trained research assistant, if they currently experienced any of the following seven symptoms: fever, headache, muscular pain, cough, a sore throat, shortness of breath, or a runny nose, or had experienced these symptoms in the 2 weeks preceding the day of the examination.

At the time of study initiation few participants of Turkish and Moroccan origin had been recruited, and therefore only participants from three ethnic groups (Dutch, Surinamese and Ghanaian) were selected for inclusion in the current study. Of participants recruited during the influenza seasons (October – March) in the years

2011 until and including March 2013, 100 symptomatic and 100 asymptomatic participants were randomly selected from each ethnic group. Participants were defined as 'symptomatic' if they reported at least either a runny nose or both fever and cough on the day of sample collection, irrespective of the presence of symptoms in the 2 weeks preceding sample collection. Participants were defined as 'asymptomatic' if they had none of the seven symptoms on the day of sample collection or in the 2 weeks preceding that day.

Patients presenting at the hospital

At the AMC, a tertiary referral centre which also provides non-tertiary care for the local area, nasopharyngeal samples are routinely collected and analysed in real time from patients clinically suspected of respiratory tract infections who are presenting at outpatient clinics or are admitted to the hospital. Types of flocked swabs and collection medium as well as the multiplex RT-PCR platform used for these routine diagnostics are identical to those used in the HELIUS population. Demographic variables and RT-PCR test results are registered in electronic registration and laboratory systems.

Anonymized demographic data and diagnostic results of patients aged ≥18 years with a clinical suspicion of respiratory tract infection, and sampled during the influenza seasons of the years 2011, 2012 and 2013, were included in the analysis.

Virological assay

Extraction of nucleic acids from 200 µl of viral transport medium was performed by MagNA Pure extraction using the total nucleic acid extraction kit (Roche Diagnostics, Penzberg, Germany). The presence of viral pathogens in samples was detected by multiplex reverse transcriptase RT-PCR as previously described [5]. Respiratory viruses detected in this assay included: rhinovirus (RV; A, B, and C), human coronavirus (hCoV: HKU1, NL63, 229E and OC43), influenza A virus (InfA), respiratory syncytial virus (RSV; A and B), influenza B virus (InfB), enterovirus (EV), adenovirus (AdV), human metapneumovirus (hMPV), parainfluenza viruses (PIVs 1, 2, 3, 4), human bocavirus (hBoV) and parechovirus (PeV).

The crossing point (C_p) value, reflecting the cycle at which a positive PCR signal is detected, was calculated using LC480 software (Roche Diagnostics, Penzberg, Germany). It was used as an approximation of the amount of viral nucleic acids

present, as C_p values are inversely correlated with the number of target DNA or RNA molecules present in the sample (in this study denoted as viral load). A difference in C_p values of 3 approximately represents a 10-fold difference in the levels of target nucleic acids. A sample was considered positive if it passed the internal positive controls and if C_p value ≤40, and negative if the value was above 40.

Statistical analysis

The study population was divided in groups according to symptom status and health care use, which was used as an approximation of illness severity, ranging from (1) asymptomatic and (2) mildly symptomatic HELIUS participants, reflective of the general population who did not seek hospital care, to three groups of symptomatic patients seeking hospital care: (3) patients presenting at general outpatient clinics, (4) patients admitted to the hospital (but not to intensive care units [ICUs]), and (5) patients admitted to ICUs. Patients presenting at the Emergency Department were excluded due to the expected high variability in severity at time of presentation within this group of patients.

Analyses of the complete study population were done to compare characteristics of individuals and prevalence of respiratory viral infections between groups (Table 1), and to assess the association between PCR-positivity and approximated illness severity based on symptom status and health care use (Table S1). In addition, among individuals with positive PCR results, we compared the distribution of viral species between groups (Table 2), and analysed possible relationships between viral load and approximated illness severity (Table 3, Figure 1, Table S2). Analyses are described in more detail below.

Differences in categorical variables were assessed using the Chi-squared test or Fisher exact test; for continuous variables Mann-Whitney U test or Kruskal-Wallis were used. Three comparisons were made in order to assess differences (1) between the symptomatic and asymptomatic HELIUS groups reflecting the general population not seeking hospital care, (2) between the three groups presenting at or admitted to the hospital, and (3) between all five groups.

Associations between groups and PCR positivity were assessed using logistic regression analysis. Because the number of positive PCR results among HELIUS

participants was small, the virus specific comparisons were restricted to the four most commonly detected viruses in the HELIUS population: RV, hCoV, InfA, and RSV. Sex, age, month and year of sampling were a priori included in the logistic regression models.

To investigate the relative distribution of viruses between groups in PCR-positive individuals, the two groups of HELIUS participants were combined (groups 1, 2) and were compared to each of the hospital groups separately (groups 3, 4 and 5) as well as combined (group 1/2 vs group 3-5). Comparisons between groups were made for each respiratory virus detected by multiplex RT-PCR.

Spearman's rank-order correlation test and linear regression analysis were done to determine relationships between viral load of RV, hCoV, InfA, RSV and approximated illness severity of groups. Both analyses were done with all groups as well as with inclusion of hospital groups only (group 3-5). Sex, age, month and year of sampling were a priori included in the linear regression models.

The level of significance in all analyses was $P<0.05$ and analyses were done in SPSS 22.0 (SPSS, Chicago, USA).

Ethics

The HELIUS study was approved by the AMC Medical Ethics Committee and informed consent was obtained from each participant at study entry. According to the Dutch law on medical research (WMO), article 1, no ethical approval is required when using anonymous data from routine diagnostic databases, as was done for the diagnostic and demographic data of AMC patients. The study was conducted according to the Dutch code of conduct for responsible use of human tissue and medical research 2011 [9].

Results

Study population

Among 3,417 HELIUS participants enrolled during the period of study, 600 adult participants were selected for inclusion in the current study. From 588 of these,

respiratory samples were available. During the same study period, respiratory samples of 600 adult patients presenting or admitted to the hospital with clinically suspected respiratory tract infection were collected and analysed. In Table 1 characteristics of the groups are reported.

The 291 asymptomatic and 297 symptomatic HELIUS participants were comparable with regard to ethnicity, education, and smoking status, but asymptomatic participants were more often male and were older. The hospital groups were comparable with regard to sex but there were significant differences between groups with regard to age (Table 1). ICU patients were older than outpatients and inpatients (both, P<0.001). HELIUS and hospital groups were not comparable with regard to gender and age (Table 1). HELIUS participants were younger (median age 49, interquartile range [IQR]=35-56) than hospital patients (median age 56, IQR=39-67) (P<0.001).

Among HELIUS participants, at least one viral pathogen was detected in 12% of participants, with a higher detection rate among symptomatic as compared to asymptomatic participants (18% versus 6%, P<0.001) (Table 1). The most prevalent viruses detected were RV (5%) and hCoV (3%), both of which were found significantly more often in symptomatic individuals. Influenza viruses were only detected in symptomatic and not in asymptomatic individuals. In 31% of patients presenting at or admitted to the hospital at least one viral pathogen was detected, and the most prevalent viruses were InfA (8%) and RV (8%). Comparisons of viral prevalence across five groups revealed significant differences for RV, InfA, RSV, InfB, hMPV, but not for the remaining viruses. Multivariable logistic regression analysis showed that the odds of viral prevalence across groups remained significantly different for RV, InfA and RSV (InfB, hMPV were not investigated) (Table S1).

There were no associations between demographic and epidemiological characteristics and PCR positivity for any respiratory virus, investigated in multivariable logistic regression analysis for each of the five groups separately (data not shown).

Table 1. Characteristics of the study population by approximated illness severity

		Asymptomatic HELIUS	**Symptomatic** HELIUS	P-value[1]	**Outpatients**	**Inpatients**	**ICU patients**	P-value[2]	P-value[3]
		n (%)*	n (%)*		n (%)*	n (%)*	n (%)*		
Total		291	297		116	284	200		
Sex									
	Male	155 (53%)	120 (40%)	0.002	63 (54%)	149 (52%)	103 (52%)	0.890	0.008
	Female	136 (47%)	177 (60%)		53 (46%)	135 (48%)	97 (49%)		
Age (years)									
	Median (IQR)	49 (40-56)	48 (32-55)	0.014[4]	53 (38-63)	52 (36-65)	63 (50-74)	<0.001[5]	<0.001[5]
	18-34	56 (19%)	81 (27%)	0.039	25 (22%)	67 (24%)	21 (11%)	<0.001	<0.001
	35-54	135 (46%)	135 (46%)		37 (32%)	85 (30%)	43 (22%)		
	≥55	100 (34%)	81 (27%)		54 (47%)	132 (47%)	136 (68%)		
Ethnicity									
	Dutch	96 (33%)	99 (33%)	0.995					
	Surinamese	96 (33%)	98 (33%)						
	Ghanaian	99 (34%)	100 (34%)						
Education (level [6])									
	1	33 (11%)	40 (14%)	0.602					
	2	89 (31%)	75 (25%)						
	3	78 (27%)	79 (27%)						
	4	90 (31%)	83 (28%)						
	Unknown	1 (0.3%)	20 (7%)						
Smoking									
	Never	174 (60%)	157 (53%)	0.630					
	Former smoker	62 (21%)	60 (20%)						
	Current	54 (19%)	60 (20%)						
	Unknown	1 (0.3%)	20 (7%)						

Table 1 (continued). Characteristics of the study population by approximated illness severity

	Asymptomatic HELIUS n (%)*	**Symptomatic** HELIUS n (%)*	P-value[1]	**Outpatients** n (%)*	**Inpatients** n (%)*	**ICU patients** n (%)*	P-value[2]	P-value[3]
Comorbidities[7]								
No	169 (58%)	120 (40%)	0.001					
Yes (CDC risk factor for resp. inf.)	86 (30%)	102 (34%)						
Yes (other)	36 (12%)	56 (19%)						
Unknown	0 (0%)	19 (6%)						
PCR positive								
For at least one virus	16 (6%)	54 (18%)	<0.001	41 (35%)	80 (28%)	66 (33%)	0.294	<0.001
Rhinovirus	6 (2%)	22 (7%)	0.002	13 (11%)	23 (8%)	13 (7%)	0.338	0.004
Human coronavirus	5 (2%)	15 (5%)	0.026	8 (7%)	12 (4%)	12 (6%)	0.490	0.082
Influenza A virus	0 (0%)	6 (2%)	0.031	4 (3%)	26 (9%)	20 (10%)	0.100	<0.001
Respiratory syncytial virus	2 (1%)	4 (1%)	0.686	8 (7%)	11 (4%)	6 (3%)	0.234	0.003
Influenza B virus	0 (0%)	1 (0.3%)	1.000	4 (3%)	4 (1%)	6 (3%)	0.346	0.001
Enterovirus	0 (0%)	1 (0.3%)	1.000	1 (1%)	0 (0%)	0 (0%)	0.193	0.214
Adenovirus	0 (0%)	1 (0.3%)	1.000	1 (1%)	1 (0.4%)	2 (1%)	0.535	0.312
Human Metapneumovirus	0 (0%)	1 (0.3%)	1.000	6 (5%)	6 (2%)	8 (4%)	0.246	<0.001
Parainfluenzavirus	1 (0.3%)	3 (1%)	0.624	2 (2%)	0 (0%)	1 (1%)	0.081	0.181
Human bocavirus	1 (0.3%)	4 (1%)	0.373	0 (0%)	1 (0.4%)	1 (1%)	1.000	0.578
Parechovirus	1 (0.3%)	1 (0.3%)	1.000	0 (0%)	0 (0%)	0 (0%)	NA	1.000
Month of sampling								
October	54 (19%)	50 (17%)	0.983	10 (9%)	28 (10%)	18 (9%)	0.002	<0.001
November	42 (14%)	41 (14%)		12 (10%)	26 (9%)	25 (13%)		
December	33 (11%)	37 (13%)		11 (10%)	20 (7%)	43 (22%)		
January	52 (18%)	58 (20%)		35 (30%)	96 (34%)	48 (24%)		
February	56 (19%)	58 (20%)		25 (22%)	58 (20%)	37 (19%)		
March	54 (19%)	53 (18%)		23 (20%)	56 (20%)	29 (15%)		

Table 1 (continued). Characteristics of the study population by approximated illness severity

		Asymptomatic HELIUS	**Symptomatic** HELIUS	P-value[1]	**Outpatients**	**Inpatients**	**ICU patients**	P-value[2]	P-value[3]
		n (%)*	n (%)*		n (%)*	n (%)*	n (%)*		
Year of sampling									
	2011	30 (10%)	42 (14%)	0.124	55 (47%)	127 (45%)	53 (27%)	<0.001	<0.001
	2012	158 (54%)	138 (47%)		34 (29%)	88 (31%)	64 (32%)		
	2013	103 (35%)	117 (39%)		27 (23%)	69 (24%)	83 (42%)		

* Unless stated otherwise

Unknown categories in *italics* were excluded from analysis.

Ethnicity, education, smoking and comorbidities were not recorded for AMC patients.

AMC= Academic Medical Center; CDC = Centers for Disease Control and Prevention; HELIUS= Healthy Life In an Urban Setting; ICU = Intensive care unit; IQR = interquartile range; PCR = polymerase chain reaction

[1] Comparison between asymptomatic and symptomatic HELIUS participants

[2] Comparison across the three AMC groups (outpatients, Inpatients, ICU)

[3] Comparison across all five groups

Chi-squared test or Fisher exact test were used for comparisons, unless stated otherwise.

[4] Mann-Whitney U-test

[5] Kruskal-Wallis test

[6] Levels were coded as:

Never been to school or elementary schooling only	1
Lower vocational or lower secondary schooling	2
Intermediate vocational or intermediate/higher secondary schooling	3
Higher vocational schooling or university	4

[7] Comorbidities known to be associated with respiratory infections [ref], and reported by HELIUS participants as being diagnosed by a physician:

Diabetes, asthma, neurological conditions, lung disease, heart disease, blood disorders, liver disorders, metabolic disorders, weakened immune system, and obesity.

Other comorbidities reported by HELIUS participants as being diagnosed by a physician included: chronic fatigue, headache or migraine, psoriasis, eczema, severe bowel disorders, arthrosis, hernia, incontinence, pain in body parts (back, neck, shoulder, elbow, hand, wrist) and chronic muscular pain.

[ref] http://www.cdc.gov/flu/about/disease/high_risk.htm

Table 2. Relative distribution of respiratory viruses in PCR-positive individuals

	HELIUS (asymptomatic and symptomatic) n (%)	**Outpatients** n (%)	**Inpatients** n (%)	**ICU patients** n (%)	**AMC patients -total-** n (%)	**p-value[1]**	**p-value[2]**	**p-value[3]**	**p-value[4]**
Total	**75**	**47**	**84**	**69**	**200**				
Rhinovirus	28 (37%)	13 (28%)	23 (27%)	13 (19%)	49 (25%)	0.271	0.180	0.014	0.035
Human coronavirus	20 (27%)	8 (17%)	12 (14%)	12 (17%)	32 (16%)	0.218	0.052	0.181	0.044
Influenza A virus	6 (8%)	4 (9%)	26 (31%)	20 (29%)	50 (25%)	1.000	<0.001	0.001	0.002
Respiratory syncytial virus	6 (8%)	8 (17%)	11 (13%)	6 (9%)	25 (13%)	0.128	0.299	0.880	0.293
Influenza B virus	1 (1%)	4 (9%)	4 (5%)	6 (9%)	14 (7%)	0.072	0.216	0.055	0.077
Enterovirus	1 (1%)	1 (2%)	0 (0%)	0 (0%)	1 (1%)	1.000	0.472	1.000	0.472
Adenovirus	1 (1%)	1 (2%)	1 (1%)	2 (3%)	4 (2%)	1.000	1.000	0.607	1.000
Human Metapneumovirus	1 (1%)	6 (13%)	6 (7%)	8 (12%)	20 (10%)	0.013	0.121	0.014	0.016
Parainfluenzavirus	4 (5%)	2 (4%)	0 (0%)	1 (1%)	3 (2%)	1.000	0.047	0.368	0.091
Human Bocavirus	5 (7%)	0 (0%)	1 (1%)	1 (1%)	2 (1%)	0.155	0.101	0.211	0.018
Parechovirus	2 (3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0.522	0.221	0.497	0.074

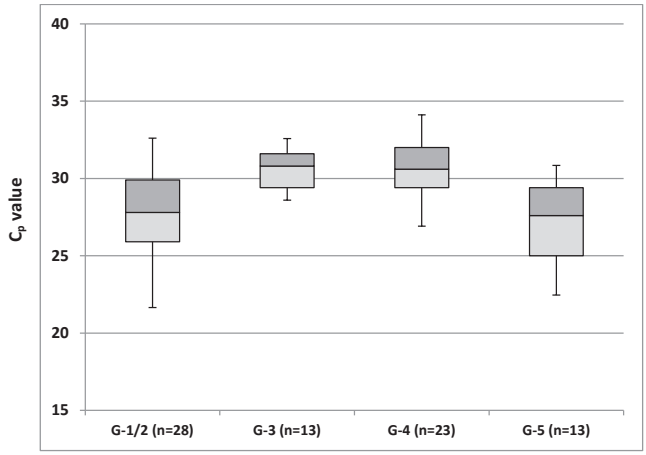
[1] Comparison between HELIUS participants and Outpatients
[2] Comparison between HELIUS participants and Inpatients
[3] Comparison between HELIUS participants and ICU patients
[4] Comparison between HELIUS participants and AMC patients (total)
Chi-squared test or Fisher exact test were used for comparisons.

AMC= Academic Medical Center
HELIUS= Healthy Life In an Urban Setting
ICU = Intensive care unit
PCR = polymerase chain reaction

1.a. Rhinovirus

Correlation between C_p values and groups arranged by approximated illness severity

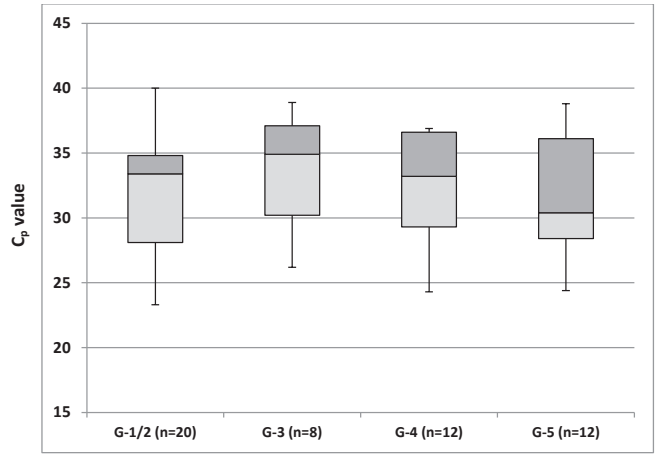
r_s = 0.112, P=0.331 (G1/2-G5)
r_s = -0.466, P=0.001 (G3-G5)



1.b. Human coronavirus

Correlation between C_p values and groups arranged by approximated illness severity

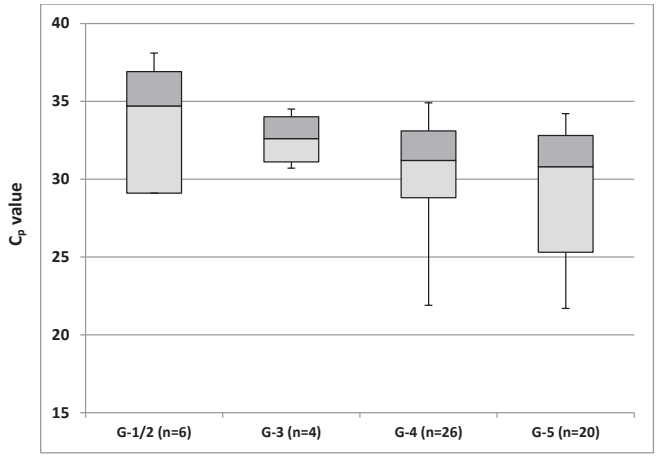
r_s = -0.032, P=0.820 (G1/2-G5)
r_s = -0.193, P=0.290 (G3-G5)



1.c. Influenza A virus

Correlation between C_p values and groups arranged by approximated illness severity

r_s = -0.311, P=0.020 (G1/2-G5)
r_s = -0.205, P=0.152 (G3-G5)



1.d. Respiratory syncytial virus

Correlation between C_p values and groups arranged by approximated illness severity

r_s = -0.374, P=0.038 (G1/2-G5)
r_s = -0.441, P=0.027 (G3-G5)

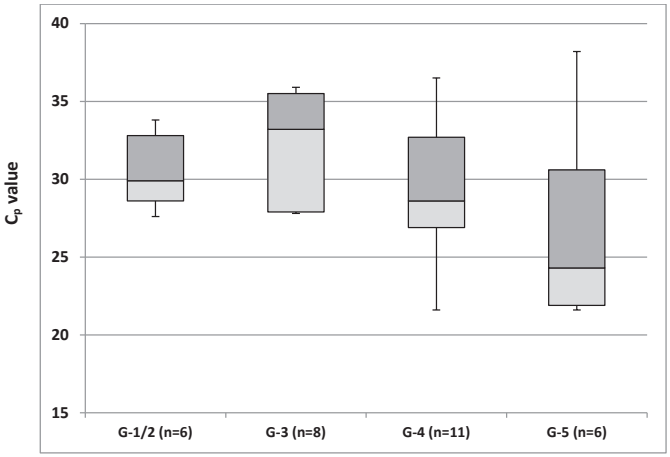


Figure 1. Box and whisker plots of crossing point values by approximated illness severity of groups

The box represents the interquartile range, the horizontal line in the box represents the median, and the whisker top and bottom represent the lowest and highest C_p values (corresponding with the highest and lowest viral load, respectively).

Please see Table 3 for median (IQR) C_p values for each group, by type of virus.

AMC= Academic Medical Center
C_p = Crossing point values
HELIUS= Healthy Life In an Urban Setting
ICU= Intensive care unit

G-1/2 = HELIUS, asymptomatic and symptomatic
G-3 = AMC, outpatients
G-4 = AMC, inpatients
G-5 = AMC, ICU patients

Relative proportions of respiratory viruses between groups

When comparing the relative proportions of specific respiratory viruses among PCR-positive individuals in each group, substantial differences were observed (Table 2). While RVs were the most commonly detected viruses in 75 PCR-positive HELIUS participants, InfA represented the largest proportion of viruses detected in 200 PCR-positive hospital patients. The proportion of RV was significantly higher among PCR-positive HELIUS participants compared to PCR-positive hospital patients (37% vs 25%, P=0.035) and was lowest among PCR-positive ICU-admitted inpatients (19%). Similarly, the proportions of hCoV and hBoV were significantly higher in HELIUS participants than in hospital patients (27% vs 16% and 7% vs 1%, respectively (P=0.044 and 0.018)). The opposite was observed for InfA and hMPV, the proportions of which were significantly higher in hospital patients compared to HELIUS participants (25% vs 8% and 10% vs 1%, respectively (P=0.002 and 0.016)) (Table 2). Highest proportions of InfA were observed in hospitalized patients (inpatients 31%, ICU inpatients 29%). Proportions of RSV and InfB were also considerably higher in hospital patients compared to HELIUS participants but these differences did not reach statistical significance.

Relation between viral load and approximated illness severity

Comparisons of viral load estimations between groups were done for the four most commonly detected viruses (RV, hCoV, InfA and RSV) as expressed by C_p values which correlate inversely with viral load levels. For RV and hCoV, no correlations of viral load were observed between the five groups (Table 3, Figure 1). Also in linear regression analysis no significant differences in hCoV load between groups were observed (Table S2). For RV, viral loads were similar between HELIUS participants and ICU-admitted patients, but significantly lower in outpatients and non-ICU

Chapter 7

General Discussion

General Discussion

In most of the tuberculosis (TB) low-incidence countries overall notification rates for TB have been declining for the last decades. Also in the Netherlands, with a total incidence of 5.1 cases per 100,000 population reported in 2013, TB has become a rare disease (1). In order to further reduce transmission rates and reach TB elimination (defined as TB incidence <1 case per million persons per year) in low-incidence countries, the WHO developed a global framework to reach these goals (2). One of the priority actions defined in this framework is to "undertake screening for active tuberculosis and latent infection in tuberculosis contacts and selected high-risk groups and provide appropriate treatment." To effectively reach this target in each country, tailored actions, depending on country-specific conditions, are required. In this thesis various aspects of epidemiological and health system conditions in the Netherlands were investigated. Here we discuss the implications of our findings, and provide recommendations for further actions and research to improve TB control in the Netherlands, and in other low-incidence countries with comparable conditions.

Recent transmission and foreign *M. tuberculosis* strain lineages

As TB incidence in the Netherlands is declining, TB elimination efforts are mainly focussed on specific high-risk groups such as the foreign-born. Rapid and accurate identification of recent transmission events is important to stop transmission chains. Clustering of *M. tuberculosis* isolates from cases with identical DNA fingerprints can be used as a proxy for recent transmission and has been a successful tool in public health to identify previously unknown transmission routes and factors associated with a higher risk of transmission (3) (4). DNA fingerprinting should therefore be used as routine surveillance to identify unexpected spread of TB and outbreaks, and to evaluate interventions (5). However, clustering as a proxy for recent transmission is not always correct, and should be interpreted with caution, especially among foreign-born cases, as isolates obtained from these patients might belong to genetically homogenous strain lineages predominating in their countries of origin. In the Netherlands, 24-locus Variable Number of Tandem Repeat (VNTR) typing was introduced in 2004, and became the new standard for DNA typing in 2009. In chapter 2 we studied the associations between VNTR genotypes of causative *M. tuberculosis* strains and their human host populations.

Main conclusions and implications

By using genotyping data of all cases diagnosed with culture-confirmed TB between October 2003 and December 2008 in the Netherlands, we found that there was a phylogeographical association between patient's origin and strain lineage. Euro-American lineages were most frequently found among non-clustered native Dutch TB cases and non-Euro-American lineages among recently arrived foreign-born cases with non-clustered *M. tuberculosis* isolates (most likely to represent importation of foreign genotypes), of whom the majority originated from Asia and Africa. Furthermore, clonal homogeneity, and reduced association of risk factors with clustering was most pronounced among non-Euro-American lineages as compared to Euro-American lineages. Together, these findings suggest a lineage-dependent degree of reliability of the inference on transmission; transmission based on clustering by VNTR typing among recently migrated foreign-born should be interpreted with caution. 24-locus VNTR typing can thus successfully be used as a public health tool to exclude transmission between individuals infected with different genotypes. However, epidemiological data are required to confirm outbreaks or transmission events when genotypes of recently immigrated foreign-born match. Collection of epidemiological data can be difficult and might be hampered by recall bias as TB patients might have been infected by a contact many years earlier, and patients of certain high risk groups, such as drug users, might be less willing to cooperate.

Further research

The clonal and phylogeographical population structure of *M. tuberculosis* emphasises the need to continuously monitor the importation of specific strain types in the Netherlands. With the growing globalization, immigration patterns in the Netherlands are subject to change, as are the strain lineages predominating in countries of origin. Characterization of predominant and emerging *M. tuberculosis* clones as part of routine surveillance will provide information on the degree of reliability of the inference on transmission for specific strain types.

Whole genome sequencing (WGS) studies will probably improve the discrimination of strains apparently identical by VNTR typing, as WGS has a greater resolution than does VNTR genotyping (6) (7). The combination of field and molecular epidemiology cannot identify who infected whom. In contrast, WGS allows inference about direction of transmission between cases, and could thus identify super-spreaders and predict the existence of undiagnosed cases, potentially leading to early treatment

of infectious patients and their contacts (6). Despite the advantages of WGS, its full public health potential remains to be investigated given the challenges of performing WGS on direct clinical material. Until then, the discriminatory power of molecular typing of *M. tuberculosis* isolates by VNTR typing could be improved by using it in combination with spoligotyping as proposed by de Beer and colleagues (8), since this has been shown to result in slightly increased discriminatory power (9) (10).

Impact of preventive TB treatment among contacts of pulmonary TB patients

To achieve TB elimination in low-incidence countries, were most cases of active TB occur due to reactivation of previously controlled latent TB infection (LTBI), health care systems have to identify TB cases at an earlier stage (2). Preventive treatment can be an effective tool to reduce the individual risk of progression to TB, and the risk of transmission to susceptible individuals in their environment. In countries where TB incidence in the general population is low, targeted testing for LTBI followed by initiation of preventive treatment, should be performed among high-risk groups (2). Therefore, contact investigations are an essential component of the tuberculosis control and elimination strategy in most low-incidence countries (11). The impact of preventive treatment is largely determined by the rate of progression to disease in the absence of preventive treatment, by the adequate identification and diagnosis of contacts with an increased risk of progression from latent infection to clinical TB, and by treatment success.

Main conclusions and implications

In chapter 3 and 4, surveillance data from the electronic system of the TB department at the GGD (Public Health Service [PHS]) Amsterdam were used to study contact investigation outcomes in the period 2002-2011. Based on these findings several opportunities to improve the impact of contact investigations on TB control in the Netherlands were identified.

In chapter 3 we found that, during 2008-2011, more than one third of the contacts of PTB patients reported to the PHS Amsterdam, who were eligible for LTBI screening, were not screened, and that about half of the contacts diagnosed with LTBI did not start preventive TB treatment. Despite the effectiveness of preventive treatment in high risk populations, suboptimal acceptance rates among both patients and

physicians have been recorded in other studies from low-incidence countries (12) (13) (14) (15). As a result, many studies have been undertaken to identify factors associated with non-acceptance (16) (17) (18). For instance, Dobler and colleagues have shown that physicians' decisions on treatment depended on the estimated risk of developing TB, which depends on the probability that a positive test result is indicative of true infection with *M. tuberculosis* (18). Thus, as a result of the increased specificity of the interferon-gamma (IFN-γ) release assay (IGRA) for detection of LTBI compared to the tuberculosis skin test (TST), it is expected that both screening coverage and initiation of preventive treatment will increase further when the IGRA is implemented as a standard diagnostic test in the Netherlands. Indeed, chapter 3 showed that, already following the introduction of the IGRA at the PHS Amsterdam in 2008, coverage of screening among contacts of PTB patients increased each year.

The impact of expanding preventive treatment depends, in addition to the acceptance, completion and efficacy of treatment, on the number of TB cases that would have been prevented if they had received treatment. Chapter 4 shows that, using 10 years of follow-up data from the electronic surveillance system on contact investigations at the PHS Amsterdam, the 5-year risk of incident TB among contacts with LTBI who did not receive preventive treatment was low at 2.4%. This chapter also showed that, even if LTBI screening and preventive treatment would have been restricted to a more selected group of contacts with increased risk of progression to TB, the 5-year risk of incident TB among contacts with LTBI who did not receive preventive treatment remained low at 3.5%. Assuming total treatment efficacy, the number of cases that would have been prevented if all would complete treatment would be low compared to the overall disease burden of 610 TB cases observed over the ten year study period in the catchment area of the PHS Amsterdam. Thus, expanding preventive treatment among TB contacts who are regarded as high risk individuals, or among a subgroup of contacts with increased risk, is unlikely to dramatically improve the population impact of preventive treatment.

In order to enhance the impact of preventive treatment in contact investigations, efforts should be taken to improve current contact tracing strategies and diagnostics tests for adequate identification and diagnosis of recently infected contacts at risk for progression to TB. As shown in chapter 4, if based on current screening strategies, targeted testing for LTBI, aimed to identify persons at high risk for TB who would benefit from treatment of LTBI, is likely to result in overtreatment. LTBI diagnosis in

this chapter was mostly by TST, as the IGRA was only recently implemented during the study period. With the increased specificity of the IGRA fewer contacts will be diagnosed with LTBI, reducing the possibility of unnecessary treatment. The ability to identify latently infected individuals at risk for developing TB would increase the clinical benefit of diagnostic tests. Previous studies have shown an association between positive IGRA results and subsequent development of TB (19) (20) (21) (22). However, these studies might have been biased as IGRA results were incorporated into the reference standard and assessments of possible TB cases were non-blinded, which could have led to relative risk estimates biased in favour of positive IGRA results (23). Indeed, Rangaka and colleagues have shown in their meta-analysis that exclusion of studies with incorporation bias resulted in only a moderate association between positive IGRA results and TB development in the included studies (23). However, these studies were all conducted in high TB incidence countries, and unbiased studies in low-incidence countries might identify increased effect measures. Nonetheless, if IGRA is to be useful as a predictive marker, studies should investigate the ability of IGRA to discriminate between individuals at risk and those who will not progress to TB, rather than demonstrating a measure of association (23). In chapter 5 we identified human biomarkers with a potential to prospectively identify individuals that develop TB. Although this study had a small sample size, was restricted to HIV-infected individuals, infection status at time of sampling was not known, and time of infection was not defined, this chapter indicated that a discriminative signature can be detected up to 6-8 months prior to clinical TB diagnosis, and these findings support the continuation of the search for predictive biomarkers. A diagnostic approach based on such biomarkers might provide the opportunity for more accurate identification of individuals with sub-clinically active *M. tuberculosis* replication and target them for preventive TB treatment, resulting in significant improvement to current treatment practices in low-incidence countries.

Further research

Prospective large scale studies will be necessary to establish the diagnostic accuracy of these biomarkers before they can be used as a diagnostic tool to identify high-risk individuals for preventive treatment or regular screening. It will be important to validate these markers in studies with longitudinal sampling in individuals with known *M. tuberculosis* infection status, in order to determine the specificity for progressive latent *M. tuberculosis* infection, and to determine the relevant time window preceding TB diagnosis. Since such studies are difficult to conduct, it will take

some time and efforts before such biomarkers can be used in a diagnostic strategy on a large scale. Short-term efforts should therefore include the collection of new evidence before scaling up LTBI screening and preventive treatment among contacts of PTB patients.

The introduction of the IGRA is unlikely to have major influence on the predictive value of contact investigations for identifying recent transmission of *M. tuberculosis*. Therefore, at the moment, the clinical benefit of a diagnostic test in contact investigations is not so much dependent on the diagnostic accuracy of the test itself, but has to rely on the ability of contact tracing strategies to identify individuals infected through a recent transmission event, and thus most likely to benefit from preventive treatment. Limited resources and the urgency of a contact investigation require prioritization of contact tracing among infectious TB patients by weighting factors associated with increased risk for recent TB infection or disease. However, previous studies have shown that lack of compliance to contact investigation guidelines is not uncommon, and that nonadherence could be the result of ambiguous recommendations, competence gaps of public health nurses, and index case-related or contact-related obstacles (24) (25) (26). Existing screening strategies might be improved if prediction models for evidence-based decision making would be used during contact investigations. Such models, using known risk factors of recent transmission, can provide estimates of the probability of a recent transmission event and inform decisions about preventive treatment (27). The utilization of such models to predict disease transmission and to aid prioritization of contact investigation is already used for selective screening of case-finding activities for sexual transmitted infections (28). Furthermore, Mamiya and colleagues have shown that it is feasible to use prediction models to estimate the probability of a newly diagnosed TB case being involved in a recent transmission chain, which can be a valuable tool in public health practice (27). These type of models can also be used to predict disease transmission among contacts of TB patients, provided that sufficient clinical outcomes of contacts are routinely captured in electronic surveillance records. For example, Chan and colleagues have shown that routinely collected data in contact investigations by public health nurses can be integrated into a predictive risk score and can help to prioritize active case finding or preventive treatment among children exposed to TB (29). Such efforts can make a significant contribution to current screening strategies, by identifying an increased proportion of contacts that will progress to TB in the absence of preventive treatment, which will improve the impact of preventive treatment in contact investigations.

However, the contribution of contact investigations to reduce the burden of disease in the population as a whole remains to be investigated. Although contacts with LTBI identified near the time of exposure are at substantial risk for the development of TB (30), all latently infected individuals, including those who acquired infection at a much earlier time, are at risk of reactivation. Most molecular epidemiological studies agreed that TB incidence among foreign-born in low-incidence countries is mainly the result of reactivation of infection acquired in country of origin (31), either in the first five years after migration (32) (33), or after extended periods of arrival (34) (35). These findings suggest low levels of transmission in low-incidence countries where most TB cases are foreign-born. Thus, even if assuming optimal contact tracing strategies and diagnostic algorithms, a large proportion of TB cases cannot be prevented by contact investigations, which aim to intervene shortly after recent transmission events have taken place. Therefore, TB control measures in low-incidence countries might benefit from shifting the focus to screening for LTBI among newly arrived immigrants and thereby achieve a significant reduction of TB rates in the population as a whole. It should be noted that the impact of immigration on the TB epidemiology in low-incidence countries is difficult to measure, among others due to incomplete strain collections and low levels of transmission (36). Some molecular epidemiological studies have suggested that some TB cases among foreign-born in low-incidence countries are the result of new infections acquired in the host country (37) (38). Therefore, it will be important to continue surveying recent transmission in low-incidence countries between foreign-born and the native population, as well as within the foreign-born communities, and adapt TB control strategies accordingly.

Clinical significance of respiratory viruses detected by RT-PCR in upper airways

Sensitive real-time polymerase chain reaction (RT-PCR) assays allow detection of a wide range of respiratory pathogens, but concerns have been raised regarding the clinical significance of respiratory viruses detected in the upper respiratory tract. PCR has not only increased viral detection in symptomatic but also in asymptomatic individuals (39) (40) (41) (42), which has made the interpretation of a positive PCR result in patients with respiratory illness challenging, and this can delay appropriate treatment initiation. Up to now, most etiological research has focused on patients, mostly children, seeking health care for acute respiratory illness, and therefore findings might not be representative of mild disease (42). Investigating respiratory

samples of upper airways by PCR in the community could provide information on the background prevalence of upper respiratory viruses and might shed light on the size of this diagnostic issue at population level.

Main conclusions and implications

In chapter 6, nasopharyngeal samples of upper airways were investigated by multiplex RT-PCR collected during the influenza seasons of 2011, 2012 and 2013, from participants in an adult population-based cohort study, reflecting the general population, and from adult outpatients and inpatients of the hospital, serving the catchment area of the cohort study population. This provided the opportunity to study the relative distribution and viral loads of respiratory viruses among adult populations by approximated illness severity, based on symptom status and health care use.

Chapter 6 showed that among virus-positive individuals, influenza virus A (InfA) and human metapneumovirus (hMPV) were overrepresented in the hospital population, while rhinovirus (RV), human coronavirus (hCoV) and human bocavirus (hBoV) were more common in the general population, confirming differences in pathogenicity between these respiratory viruses. Combined, InfA and hMPV contributed to 35% of detected viruses in the hospital population versus only 9% in the general population, suggesting that causality can be implied if detected in patients presenting with acute respiratory illness. However, this was less straightforward for viruses such as RV, hCoV and hBoV, which together represented 42% of detected viruses in the hospital population, indicating that positive PCR results of such viruses should be interpreted with caution if detected in patients with respiratory symptoms seeking health care. Additional interpretative parameters for such viruses circulating in the community could make a significant contribution to the clinical interpretation of diagnostic results in hospitalized patients. Viral load might represent one such parameter. However, in chapter 6, a significant relation between levels of viral replication and approximated illness severity, was found only for InfA, while these correlations were less clear for RV, hCoV, for which additional parameters are most needed.

Although we found a relation between InfA viral load and approximated illness severity, mere PCR detection of InfA was already a strong indicator of the causality between infection and symptoms, suggesting that viral load as an additional interpretative parameter is of little added value to qualitative PCR results. Nevertheless, InfA viral

load might have prognostic value given its relation with illness severity. In addition, viral load measurements may be important to monitor the effect of antiviral therapy and detect treatment failure, e.g. due to development of drug resistance, early in course of treatment.

Few other studies investigated associations between quantitative PCR results of viral pathogens and illness severity in a study population consisting of both asymptomatic and symptomatic individuals. These studies show conflicting results. For instance, Jansen and colleagues also found higher InfA viral loads in symptomatic patients than in asymptomatic controls, but, in contrast to our findings, they also found significant higher RV and hCoV viral loads among symptomatic individuals (40). Fuller and colleagues did not find any association between InfA loads and illness severity (43). These contradicting results might be due to the lack of validated definitions of illness severity, usage of different PCR assays, or could be the result of heterogeneous study populations, e.g. our study population consisted of adults while most etiological studies, including the study by Jansen and colleagues, focus on children (40) (44).

Further research

Prospective studies with larger sample sizes and inclusion of different spectra of respiratory disease and infectious etiologies are clearly needed before viral load can be used as an additional interpretive parameter in clinical practice. Given the obvious need for improved diagnostics, it might be more rewarding to search for other parameters of pathogenicity to assist patient classification. Detailed analysis of the host response to infection by different pathogens could be such an alternative approach (45). Each infectious agent interacts with specific pattern-recognition receptors differentially expressed on human blood leukocytes, which thus constitute an accessible source of clinically relevant information (46). In fact, recent transcriptional profiling studies have demonstrated pathogen-specific gene-expression profiles detected in the peripheral blood of patients with acute infections (45) (47) (48). Gene-expression profiling can increase our knowledge on the pathogenesis of infections and biomarkers of disease severity, and thus might be a suitable candidate to assist in treatment decisions (46).

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Chapter 8

Summary
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Summary

The studies described in this thesis assessed the molecular epidemiology of tuberculosis (TB) among the Dutch population, and the impact of contact investigation regarding TB prevention in high-risk groups. In addition, it was investigated if biomarkers can be identified that predict which individuals will progress to TB within a high TB risk population. Finally, the prevalence, relative distribution and viral load of respiratory viruses were compared among adults in the general population and among adult outpatients and inpatients of the hospital serving the catchment area of the general population.

Chapter 1 provides an overview of the epidemiology of TB and respiratory viruses, and describes concepts that are relevant for TB control. This chapter also presents the outline of the studies described in this thesis.

In **chapter 2** we investigated whether 24-locus Variable Number of Tandem Repeat (VNTR) typing is suitable to identify recent transmission between TB cases by using genotyping data of all cases diagnosed with culture-confirmed TB in the period 2003-2008 in the Netherlands. This study provided evidence of a lineage-dependent degree of reliability of the inference on transmission. Clonal homogeneity and reduced association of risk factors with clustering, as proxy for recent transmission, was most pronounced among non-Euro-American lineages as compared to Euro-American lineages. We concluded that transmission based on clustering by VNTR typing among recently migrated foreign-born people should be interpreted with caution as non-Euro-American lineages were most frequently found among recently arrived foreign-born cases.

In c**hapter 3** and **4**, surveillance data from the electronic system of the TB department at the GGD (Public Health Service [PHS]) Amsterdam were used to study contact investigation outcomes in the period 2002-2011.

In **chapter 3** the success of TB contact investigations was evaluated by investigating compliance to national guidelines and by determining its coverage and yield in the period 2008-2011. We found that more than one third of the contacts of pulmonary TB (PTB) patients reported to the PHS Amsterdam, who were eligible for latent tuberculosis infection (LTBI) screening, were not screened, and that about half of the

contacts diagnosed with LTBI did not start preventive TB treatment. Furthermore, we found that, following the introduction of the interferon-gamma (IFN-γ) release assay (IGRA) at the PHS Amsterdam in 2008, coverage of LTBI screening among contacts of PTB patients increased each year. We recommended that LTBI screening should be further expanded, especially among BCG-vaccinated contacts and that future research should investigate factors associated with the uptake of preventive treatment for both physicians and patients.

Chapter 4 investigated the potential impact of preventive TB treatment among contacts of PTB patients by estimating the risk of TB among latently infected contacts according to initiation of preventive treatment using 10 years of follow-up data. This study showed that the 5-year risk of incident TB among contacts with LTBI who did not receive preventive treatment was low at 2.4%, and that even if LTBI screening and preventive treatment would have been restricted to first ring contacts of PTB patients, the 5-year risk remained low at 3.5%. These findings suggested that limited impact may be expected of expanding preventive treatment. Furthermore, this study indicated the need to reassess cost-effectiveness estimates of preventive TB treatment in low TB incidence countries, as these studies generally used higher estimates of TB activation.

In **chapter 5** we investigated whether human biomarkers can be identified to predict which individuals will progress to TB. For this study we used retrospectively selected blood samples of HIV-infected drug users and compared gene expression profiles in samples of drug users months before clinical TB diagnosis with gene expression in samples of drug users who did not develop TB. Although this study had a small sample size, was restricted to HIV-infected individuals, infection status at time of sampling was not known, and time of infection was not defined, it provided evidence that a discriminative signature can be detected months prior to clinical TB diagnosis. These findings support the continuation of the search for predictive biomarkers. We therefore recommended that future studies should repeat these analysis on larger, well defined cohorts.

The aim of **chapter 6** was to better understand the clinical significance of respiratory viruses detected by sensitive real-time polymerase chain reaction (RT-PCR) assays. In this chapter we compared the prevalence, relative distribution and viral load of respiratory viruses among a random sample of participants in an adult population-

based cohort study, reflecting the general population, and among adult outpatients and inpatients of the hospital serving the catchment area of the cohort study population. This provided the opportunity to study the relative distribution and viral loads of respiratory viruses among adult populations by approximated illness severity, based on symptom status and health care use. We found that among virus-positive individuals, influenza virus A and human metapneumovirus were overrepresented in the hospital population, while rhinovirus, human coronavirus and human bocavirus were more common in the general population, confirming differences in pathogenicity between these respiratory viruses. Viral load correlated with illness severity for influenza virus A and respiratory syncytial virus but this was less clear for viruses with reduced pathogenicity, like rhinovirus and human coronavirus. We therefore concluded that the clinical value of such viruses detected by PCR remains difficult to determine, and that detailed prospective studies across the different spectra of disease and infectious etiologies are clearly needed to further our understanding.

Chapter 7 reviewed the main findings and implications of these studies and provided recommendations for improving TB control in the Netherlands and other low TB incidence countries. In essence this thesis showed that a large proportion of TB cases in the Netherlands cannot be prevented by contact investigations, arguing against the expansion of preventive treatment among TB contacts. It was recommended that it should be investigated whether TB control measures might benefit from shifting the focus to screening for LTBI among newly arrived immigrants and thereby achieve a significant reduction of TB rates in the population as a whole. To accurately measure the impact of immigration on TB epidemiology in the Netherlands and adjust control strategies accordingly, it will be important to continue surveying recent transmission between and within the foreign-born and native populations. This thesis provided evidence that recent transmission based on identical VNTR patterns of certain *M. tuberculosis* strain types should be interpreted with caution. Therefore, monitoring the importation of predominant and emerging *M. tuberculosis* clones should be part of routine surveillance on recent transmission of TB in the Netherlands.

Samenvatting

De onderzoeken in dit proefschrift geven een beschrijving van de moleculaire epidemiologie van tuberculose (TB) in de Nederlandse bevolking, en bestuderen de opbrengst van contactonderzoek en de incidentie van TB na het contactonderzoek. Daarnaast is onderzocht of er biomarkers geïdentificeerd kunnen worden die het risico op progressie van infectie naar TB kunnen voorspellen in een populatie met een hoog risico op TB. Tot slot zijn de prevalentie, relatieve verdeling en virus hoeveelheden van respiratoire virussen in monsters van de bovenste luchtwegen vergeleken tussen volwassen populaties afkomstig uit de algemene bevolking en het ziekenhuis.

Hoofdstuk 1 geeft een overzicht van de epidemiologie van TB en respiratoire virussen, en beschrijft aspecten die relevant zijn voor de tuberculosebestrijding. Dit hoofdstuk presenteert ook de hoofdlijnen van de in dit proefschrift beschreven onderzoeken.

In **hoofdstuk 2** hebben we onderzocht of 24-locus Variable Number of Tandem Repeat (VNTR) typering geschikt is om recente transmissie tussen TB patiënten te identificeren. Hiervoor hebben we gebruik gemaakt van typeringsdata van alle TB patiënten die in de periode 2003-2008 in Nederland gediagnosticeerd waren met bacteriologisch bevestigde TB. Dit onderzoek heeft laten zien dat de betrouwbaarheid van VNTR om recente transmissie vast te stellen afhankelijk is van het genotype. TB stammen die behoren tot de non-Euro-American genotypes leken onderling veel sterker op elkaar dan de stammen die tot de Euro-American genotypes behoren. Daarnaast nam de associatie van risicofactoren met clustering, als maat voor recente transmissie, sterk af wanneer de analyse beperkt werd tot de stammen die behoren tot de non-Euro-American genotypes ten opzichte van de sterkte van deze associaties in de analyse met de stammen die tot de Euro-American genotypes behoren. We concludeerden dat transmissie geïdentificeerd door VNTR typering voorzichtig geïnterpreteerd moet worden bij recent aangekomen immigranten vanwege de relatief hoge prevalentie non-Euro-American genotypes die wij vonden in deze populatie.

In **hoofdstuk 3** en **4** zijn surveillance data uit het elektronisch systeem van de TB afdeling van de Geneeskundige en Gezondheidsdienst Amsterdam (GGD) Amsterdam gebruikt om uitkomsten van het contactonderzoek in de periode 2002-2011 te bestuderen.

In **hoofdstuk 3** hebben we het succes van de TB contactonderzoeken in de periode 2008-2011 geëvalueerd door onderzoek te doen naar de dekkingsgraad en de opbrengst van het contactonderzoek, en in hoeverre richtlijnen in het contactonderzoek worden nageleefd. We vonden dat meer dan één derde van de contacten van pulmonale TB (PTB) patiënten gerapporteerd aan de GGD Amsterdam, die in aanmerking kwamen voor screening van latente tuberculose infectie (LTBI), niet gescreend waren, en dat ongeveer de helft van de contacten gediagnosticeerd met LTBI niet met preventieve TB behandeling gestart was. Dit onderzoek liet ook zien dat, na de introductie van de interferon-gamma (IFN-γ) release assay (IGRA) bij de GGD Amsterdam in 2008, de dekkingsgraad van LTBI screening onder contacten van PTB patiënten elk jaar toenam. We deden de aanbeveling dat LTBI screening verder uitgebreid zou moeten worden, met name onder BCG-gevaccineerde contacten. Toekomstig onderzoek zou zich moeten richten op het identificeren van factoren die geassocieerd zijn met de acceptatie van preventieve TB behandeling onder zowel behandelend artsen als patiënten.

Hoofdstuk 4 onderzocht de potentiele impact van preventieve TB behandeling onder contacten van PTB patiënten. Hiervoor hebben we het risico op TB geschat onder contacten met LTBI die al dan niet met behandeling startten, waarbij we gebruik maakten van follow-up data die een 10-jarige periode besloegen. We vonden dat het 5-jaars risico op incidente TB onder contacten met LTBI die geen behandeling waren gestart laag was met 2,4%, en dat, zelfs wanneer het onderzoek zou zijn beperkt tot de eerste ring contacten van PTB patiënten, het 5-jaars risico laag bleef met 3,5%. Deze bevindingen suggereren dat uitbreiding van preventieve behandeling een beperkte impact zal hebben. Daarnaast laten onze resultaten zien dat schattingen van de kosteneffectiviteit van preventieve TB behandeling in landen met een lage TB incidentie mogelijk herzien moeten worden, aangezien deze onderzoeken over het algemeen hogere schattingen van het risico op TB hanteerden.

In **hoofdstuk 5** is gekeken of we biomarkers konden identificeren die kunnen voorspellen welke individuen TB ontwikkelen. Voor dit onderzoek hebben we retrospectief bloedmonsters van HIV-geïnfecteerde druggebruikers geselecteerd en hebben we de genexpressieprofielen van de druggebruikers die geen TB ontwikkelden vergeleken met de genexpressieprofielen van de druggebruikers waarbij wel TB gediagnosticeerd was. De bloedmonsters van deze laatste groep waren afgenomen in de maanden voorafgaand aan de klinische TB diagnose. Hoewel dit onderzoek uit een

kleine studiepopulatie bestond, beperkt was tot HIV-geïnfecteerden individuen, de infectiestatus onbekend was, en het tijdstip van infectie niet gedefinieerd was, lieten de resultaten zien dat er biomarkers zijn met voldoende onderscheidend vermogen om te voorspellen welke individuen TB ontwikkelen in de maanden voorafgaand aan de klinische diagnose. Daarom adviseerden we om deze analyses te herhalen in een groter cohort met longitudinale sampling, onder individuen met verschillende comorbiditeiten en van wie de infectiestatus en het tijdstip van infectie bekend zijn.

Hoofdstuk 6 had als doel om meer inzicht te krijgen in de klinische relevantie van respiratoire virussen gedetecteerd door gevoelige real-time polymerase kettingreactie (RT-PCR) testen. In dit hoofdstuk zijn de prevalentie, relatieve verdeling en virus hoeveelheden in luchtwegmonsters van respiratoire virussen vergeleken tussen volwassenen afkomstig uit een steekproef van de algemene bevolking en volwassen patiënten die zich tijdens dezelfde periode, gedurende de influenza seizoenen in de periode 2011-2013, presenteerden of waren opgenomen in het AMC; in het verzorgingsgebied van dit ziekenhuis woont de populatie van waaruit de steekproef uit de algemene bevolking was genomen. Dit gaf ons de mogelijkheid om de relatieve verdeling en virus hoeveelheden van respiratoire virussen te bestuderen in luchtwegmonsters verkregen van volwassen populaties die verschilden in gezondheid, geschat op basis van symptoomstatus en zorggebruik. Onze resultaten lieten zien dat onder de PCR-positieve individuen, het influenza A virus en het humaan metapneumovirus oververtegenwoordigd waren in de ziekenhuispopulatie, terwijl het rhinovirus, het humaan coronavirus en het humaan bocavirus frequenter voorkwamen in de algemene bevolking. Deze bevinding bevestigt verschillen in virulentie tussen deze virussen. De virus hoeveelheden van het influenza A virus en het respiratoir syncytieel virus in luchtwegmonsters waren gecorreleerd met de gezondheidstoestand van de studiepopulaties, maar deze relatie was minder duidelijk voor minder virulente virussen, zoals het rhinovirus en het humaan coronavirus. We concludeerden daarom dat het lastig blijft om bij individuele patiënten de klinische relevantie te bepalen van een positief PCR resultaat van dit soort virussen, en dat gedetailleerd prospectief onderzoek in populaties met een breed klinisch spectrum aan respiratoire klachten en infecties nodig is om de klinische interpretatie van PCR resultaten te verbeteren.

In **hoofdstuk 7** bespreken we de belangrijkste bevindingen van de onderzoeken in dit proefschrift en geven we suggesties met betrekking tot het verbeteren van de

tuberculosebestrijding in Nederland en in andere landen met een lage TB incidentie. Het onderzoek heeft laten zien dat een groot deel van de TB patiënten in Nederland niet voorkomen kan worden door contactonderzoek, dit suggereert dat uitbreiding van preventieve TB behandeling onder TB contacten hier geen verandering in zal brengen. Onderzoek zal moeten uitwijzen of het screenen op infectie van immigranten bij binnenkomst in Nederland zal resulteren in een significante reductie van de TB incidentie in de algemene populatie. Om de impact van immigratie op de epidemiologie van TB in Nederland accuraat te kunnen meten, is het van groot belang om recente transmissie tussen en binnen de migranten populaties en de Nederlandse bevolking continu te blijven monitoren. We hebben laten zien dat identieke VNTR patronen als maat voor recente transmissie bij bepaalde TB genotypes voorzichtig geïnterpreteerd moet worden. Daarom adviseren we om de import van TB stammen nauwgezet te monitoren als onderdeel van de routine surveillance naar recente transmissie van TB in Nederland.

Dankwoord

Misschien wel het belangrijkste deel van mijn proefschrift, het dankwoord. Veel mensen hebben bijgedragen aan de totstandkoming van dit proefschrift. Daarom wil ik hen graag bedanken.

Als eerste gaat mijn dank uit naar prof. dr. Borgdorff. Beste Martien, ik heb grote bewondering voor jouw kennis en scherpe blik. Wanneer ik weer eens wat moeilijk dacht kon jij altijd heel helder verwoorden hoe de vork in de steel zat, waardoor ik weer met frisse moed vooruit kon. Ik ben dan ook absoluut van mening dat ik mij geen betere promotor had kunnen wensen! Bedankt voor alles.

Dr. Schim van der Loeff, beste Maarten, ondanks dat je pas in een later stadium mijn copromotor bent geworden, was je altijd zeer betrokken bij mijn promotieonderzoek. Niet alleen voorzag jij mijn stukken heel snel van commentaar, dankzij jou klopte alles ook tot in het kleinste detail. Bedankt voor je toegankelijkheid en de prettige samenwerking.

Prof. dr. de Jong, beste Menno, promotor, dankzij jouw vele bruikbare ideeën en rake opmerkingen heb ik ook het laatste deel van dit traject tot een mooi einde kunnen brengen. Veel dank daarvoor!

Geachte leden van de promotiecommissie, hartelijk dank voor het beoordelen van mijn proefschrift en deel te zijn van de promotiecommissie. Ik weet dat het een aanslag op uw schaarse tijd is en waardeer het daarom des te meer.

Prof. dr. van Soolingen, beste Dick, graag bedank ik jou voor de fijne samenwerking aan het begin van mijn promotie. Ik vond onze gesprekken altijd erg inspirerend. Je enthousiasme, vele ideeën, en alles wat je me bijbracht over de moleculaire epidemiologie van tuberculose, maakte dat ik altijd met veel plezier bij het RIVM op bezoek kwam. Bedankt daarvoor en voor alle vrijheid die je mij in mijn onderzoek hebt gegeven.

Alle coauteurs wil ik bedanken voor hun bijdrage aan de verschillende studies. Beste Simone, in het bijzonder dank ik jou voor de leuke samenwerking. Ik heb veel geleerd van jouw immunologische kennis die je met mij wilde delen. Daarnaast heb

ik bewondering voor je altijd pragmatische aanpak gedurende onze samenwerking. Beste Jessica, bedankt voor onze leuke brainstormsessies op het RIVM. Wat vulden wij elkaar mooi aan. Ik wens je heel veel succes met alles!

Beste Marieke en Louise, wat bijzonder was het om samen met jullie en anderen zo aan het begin van HELIUS te hebben gestaan, en wat een werk is er daarna nog verricht. Mooi om te zien hoe HELIUS is uitgegroeid tot zo'n groot cohort. Louise, super fijn om met jou tussendoor op het AMC of daarbuiten te kunnen sparren over van alles. Leuk dat we nu ook tegelijkertijd klaar zijn. Heel veel succes straks! Beste medewerkers van de Medische Microbiologie, bedankt voor al jullie werk voor HELIUS.

Stafleden en KEBB-aio's bedankt voor alles. Prof. dr. Zwinderman, beste Koos, ik wil je bedanken voor het altijd prettig contact en je toegankelijkheid. Jouw deur stond altijd open. Daarnaast kunnen maar weinig mensen zeggen dat ze samen met hun baas aan een hardloopwedstrijd hebben meegedaan! Michel, bedankt voor al je hulp wanneer ik weer eens met vragen over *R* kwam aanzetten. Jammbe, Erik, Iris, Parvin, Annefloor, Umesh en Eleonor bedankt voor jullie gezelligheid. Beste Annette, veel dank voor al je hulp.

Beste GGD collega's van de Tuberculose afdeling, bedankt voor jullie gastvrijheid en behulpzaamheid. In het bijzonder wil ik Peter en Sytze bedanken voor het meedenken over onze onderzoeksresultaten. Heel fijn dat ik met vragen altijd bij jullie terecht kon. Ik heb veel geleerd van jullie klinisch inzicht. Annet, bedankt dat je mij wegwijs hebt gemaakt in de wereld van de contactonderzoeken. Beste Carolien, bedankt voor al je hulp met TUBIS.

Beste GGD collega's van de afdeling Infectieziekten, toen ik met Martien mee verhuisde naar de GGD voelde ik mij direct thuis bij jullie. De bijzondere sfeer op de afdeling en diversiteit aan persoonlijkheden maakte dat ik een ontzettend leuke tijd bij jullie heb gehad! Veel dank daarvoor. Janneke, Martijn en Daniëla bedankt voor alle gezelligheid in de 30+ kamer waarin ik – ondanks dat ik deze memorabele leeftijd nog niet heb bereikt – vooral tegen het einde van mijn promotie veel tijd heb doorgebracht. Weer of geen weer, in de pauzes waren jullie gelukkig altijd in voor een wandeling in de buitenlucht! Dani, welk vega-restaurant moeten we nog van ons lijstje strepen? Rik, Frank, en overige MudMaster-fanaten, bedankt voor de leuke trainingen in het Oosterpark en Amsterdamse Bos. Reinier, veel dank voor je hulp met

BioNumerics, en voor alle leuke gesprekken over ons onderzoek, en nog veel meer. Sofie, wat gezellig om jou ook buiten de GGD beter te leren kennen, ik hoop dat we onze etentjes blijven voortzetten. Wijnand, Wendy, Gerben Rienk, Lisanne, Camiel, Marjolein, Marc, Christiaan, Bart, Joost, Freke, Carolien, Linda, Nora, Maaike, Nicol, Eline, Jannie, Amy, Titia, Udi, Ineke, Ronald, Robin, Maria, Rianne, Alfons, Menne, Susanne, Marleen, Bart-Jan en nog vele anderen, bedankt voor alle gezelligheid!! Will, ontzettend bedankt voor al je hulp, maar vooral voor alle leuke gesprekken op de gang. Ik hoop dat we contact blijven houden.

Heel blij ben ik met mijn lieve vriendinnen die absoluut de perfecte ontspanning zijn geweest tijdens mijn promotietijd. Fleur, het duurde even maar ik ben klaar, we kunnen weer de dansvloer op! Lotte & Marianne, ik leerde jullie kennen in het moppen-hok, heel leuk dat ons contact is gebleven. Eva, ondanks dat ik uiteindelijk toch koos voor hardlopen in plaats van mij te laten afbeulen tijdens Bootcamp, ben ik blij dat we onze gesprekken vrolijk hebben voortgezet onder het genot van vele wijntjes. Ook niet verkeerd.

Lieve Nienke, heel gaaf dat je mijn paranimf wilt zijn! Ik weet nog toen wij elkaar voor het eerst zagen bij een HELIUS PG meeting. Maria dacht dat we elkaar al jaren kende en zo voelde dat ook wel een beetje. Heerlijk dat je altijd zegt waar het op staat, met veel inzicht en humor. Bedankt voor je gezelligheid en goede gesprekken. Wanneer gaan we weer wielrennen? Mag ook een rustig klets-rondje zijn hoor!

Carlijn, Esther en Inge, de VU ligt alweer een tijdje achter ons, maar gelukkig zijn we nog steeds dikke matties. Afzien tijdens de 150 km Amstel Gold Race, tripjes naar Londen, en natuurlijk de oneindig veel etentjes en borrel momenten in de leukste stad van de wereld. Ik geniet altijd van jullie humor en gezelligheid. Es, jij bent next! Heel fijn om af en toe met jou via Skype te kletsen over onze promotie-perikelen en prestaties op hardloopgebied. Gezellig dat je nu wat vaker in Amsterdam bent te vinden. En anders komen wij gewoon weer naar Londen. Als echte John Snow Society members moeten we binnenkort toch echt eens een meeting bijwonen.

Lieve Lenneke en Coralie, al zo'n 15 jaar zijn jullie mijn vriendinnetjes. Ik zou een proefschrift vol kunnen schrijven over wat jullie voor mij betekenen. Soms lijkt het wel alsof jullie mij beter kennen dan ik zelf. Bedankt dat jullie er altijd voor mij zijn, en voor de onvoorwaardelijke vriendschap. Wat ben ik een bofkont met twee van die toppers!

Ook wil ik graag mijn schoonfamilie bedanken, lieve Mieke en Rob, bedankt voor jullie belangstelling. Ik heb mij altijd zeer welkom gevoeld bij jullie thuis!

Lieve pap en mam, bedankt dat jullie mij zoveel moois hebben bijgebracht en nog steeds bijbrengen. Dankzij een positieve levenshouding gaven jullie mij de beste omgeving om in op te groeien! Ik waardeer het enorm dat jullie er altijd voor mij zijn, zelfs als we ons weer in verschillende uithoeken van de wereld begeven. Lieve Job en Emy, wat ben ik blij met jullie als broertje en zusje! We zijn zo verschillend en toch ook weer zo hetzelfde. Aan een paar woorden hebben we vaak al genoeg. Bij jullie zijn voelt dan ook echt als thuiskomen.

Mijn laatste woorden zijn voor jou lieve Paul. Ik ben trots dat ik jou al vele jaren mijn vriend mag noemen, en ontzettend dankbaar voor alle bijzondere momenten die wij samen al hebben beleefd. We hebben prachtige reizen gemaakt en nog zoveel mooie plannen! Dank voor je humor, steun en begrip. Ik kijk uit naar alle avonturen die nog komen gaan!

About the Author

Rosa Sloot was born on 26 Oktober 1985 in Haarlem, the Netherlands. After completing her pre-university education at the Jac. P. Thijsse College in Castricum, she started her studies in 2004 at the VU University Amsterdam. After obtaining her bachelor's degree in Health Sciences in 2007, Rosa started her master's degree in Infectious Diseases and Public Health. She completed her internship at the Centre for Infectious Disease Control (CIb) of the National Institute for Public Health and the Environment (RIVM) in Bilthoven, where she evaluated the risk-based visitor-prioritizing system at the sexually transmitted infection clinics in the Netherlands. In 2008 she received her master's degree and subsequently she started her second master's degree in Biomedical Sciences, differentiation Public Health Research. Rosa conducted her master's thesis on the reproducibility of T-cell-based assays for tuberculosis infection at the Department of Infectious Diseases, Oregon Health and Science University (OHSU), Portland, United States of America.

After completing her studies in 2009, Rosa worked as a Junior Lecturer at the department of Health Sciences, VU University Amsterdam. In 2010 she started as a PhD candidate at the Academic Medical Centre of Amsterdam (AMC), University of Amsterdam, and was positioned at the GGD Amsterdam. Her PhD work consisted of several epidemiological studies which intended to improve TB control in the Netherlands and other low TB incidence countries. In addition, she assisted in the establishment of the HELIUS study, a multi-ethnic prospective cohort study.

Portfolio

AMC Graduate School for Medical Science – PhD Portfolio

Name PhD student:	Rosa Sloot
Names PhD supervisors:	Prof. dr. M.W. Borgdorff, Prof. dr. M.D. de Jong, dr. M.F. Schim van der Loeff

Courses 2010- 2013

AMC Graduate School

- Advanced Topics in Biostatistics
- Advanced Topics in Clinical Epidemiology
- Crash Course Basic Chemistry
- The AMC World of Science

Netherlands Institute for Health Sciences (NIHES) Rotterdam

- Survival Analysis
- Epidemiology of Infectious Diseases

Presentations

- Union World Conference on Lung Health, 2014, Barcelona; poster discussion session [presented orally] entitled: 'Risk of tuberculosis: a 10-year follow-up study of contacts in Amsterdam'.
- European Congress of Clinical Microbiology and Infectious Diseases, 2013, Berlin; poster presentation entitled: 'The cluster definition in variable number of tandem repeat (VNTR) typing in relation to the population structure of *Mycobacterium tuberculosis* in the Netherlands'.

Supervision of students

Supervised student on her Masters of Science thesis for her study Health Sciences, differentiation Infectious Diseases, at the VU University.

List of publications

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