The detection and prevention of airborne tuberculosis transmission

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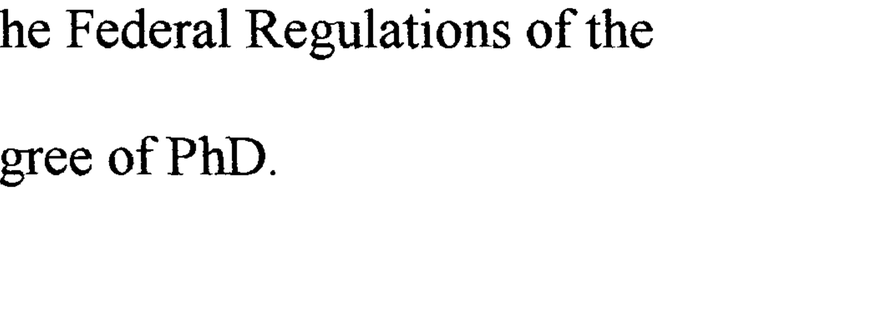
DECLARATION

No part of this dissertation is the same as any that I have submitted for a degree, diploma, or any qualification at this or any other university.

This thesis meets the requirements stipulated by the Federal Regulations of the University of London for the Degree of PhD.

The work described in this dissertation is the result of my own work, except where otherwise stated.



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also came from Roxana Anglats Mori, who performed approximately 10% of the autopsies. The remaining autopsies were performed by the PhD candidate, who also personally supervised >95% of all autopsies. A number of people assisted in the skin testing process, and Miguel Gil and Roxana Anglats assisted in the siting of the guinea pig PPD skin tests and the reading of the skin tests in quarantine. All PPD skin tests in guinea pigs exposed to ward air were read by the PhD candidate. Only a small proportion of the samples of guinea pig organs were homogenised and cultured for *M. tuberculosis* by the PhD candidate. The majority were carried out by staff at the Laboratorio de Investigación y Desarrollo, in particular Luz Caviedes, Pilar Navarro, Cesar Jerí, Willi Quino, Teresa Valencia and Beatriz Herrera. Processing of all patient sputum samples and all TB drug sensitivity testing was carried out as part of the routine service at our research laboratory, the Laboratorio de Investigación y Desarrollo, and none were processed by the PhD candidate. Approximately one half of the DNA extraction and spoligotyping analysis of patient and guinea pig TB strains was carried out by Fanny Arenas, Patricia Sheen, and Melissa Méndez, the remainder being performed by the PhD candidate. Approximately one half of patient admission questionnaires and the majority of daily symptom questionnaires were conducted by research nurses Rosa Yataco and Monica Peralta, who also supervised the majority of patient sputum collection at Hospital Nacional Dos de Mayo.

To Mum and Dad

SUMMARY

Institutional transmission of tuberculosis is an important public health problem, especially in low resource settings where protective measures such as negative-pressure isolation rooms are difficult to implement. Natural ventilation by simply opening windows and doors is recommended by the World Health Organisation for tuberculosis control in resource limited settings, but efficacy is poorly defined.

In the first part of this thesis, the rates and determinants of natural ventilation were investigated in a wide variety of clinical settings in 8 hospitals in Lima, Peru. Natural ventilation was measured in 70 clinical rooms using a carbon dioxide tracer-gas technique, compared with 12 mechanically ventilated negative-pressure rooms. Results were used to model tuberculosis transmission risk. Natural ventilation by opening windows and doors resulted in high rates of air exchange, especially in old-fashioned wards with high ceilings and large windows. Modelling predicted reduced tuberculosis transmission with natural ventilation compared with mechanical ventilation. However, natural ventilation is not appropriate in cold climates and other environmental controls are needed. Evaluation of such controls is complicated by the difficulty of measuring *M. tuberculosis* in air. Therefore the second part of this thesis deals with the creation and optimisation of a guinea pig air sampling facility to detect airborne tuberculosis transmission. Situated above a tuberculosis ward, an average 92 guinea pigs over 16 months breathed exhaust air from the ward which was occupied by 97 patients co-infected with HIV and pulmonary tuberculosis. DNA fingerprinting of animal and patient tuberculosis strains demonstrated great heterogeneity of patient infectiousness, tuberculosis transmission from a minority of patients, and highly infectious individual multidrug-resistant TB-HIV cases. This guinea pig air sampling model is now in use evaluating upper room ultraviolet light and negative air ionisation for reducing institutional tuberculosis transmission.

ABBREVIATIONS

ACH	air-changes per hour
AIDS	acquired immune deficiency syndrome
CD4	cluster of differentiation 4 (T helper cell)
cfm	cubic feet per minute
cfu	colony forming units
CI	confidence interval
CO_2	carbon dioxide
dATP	deoxy-adenine triphosphate
dCTP	deoxy-cytidine triphosphate
dGTP	deoxy-guanosine triphosphate
DNA	deoxyribonucleic acid
DOTS	directly observed therapy, short course
dTTP	deoxy-thymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
HCW	health care worker
HEPA	high efficiency particulate air
HIV	human immunodeficiency virus
INS	Instituto Nacional de Salud
IRB	Institutional Review Board
M.	Mycobacterium
MDR-TB	multidrug-resistant tuberculosis
mRNA	messenger ribonucleic acid
NALC	N-acetyl-L-cysteine-sodium hydroxide
N95	≥95% filter efficiency for particles of 0.3 μm diameter and resistant to degradation by oil based aerosols
PCR	polymerase chain reaction
PPD	purified protein derivative
ppm	parts per million
rpm	revolutions per minute
SD	standard deviation
SDS	sodium dodecyl sulphate
TB	tuberculosis
TE	Tris-HCl:EDTA:Triton
TEMA	tetrazolium microplate assay
Tris	tris(hydroxymethyl)aminomethane
UPCH	Universidad Peruana Cayetano Heredia
UV	ultraviolet
UVGI	ultraviolet germicidal radiation
v/v	volume for volume
w/v	weight for volume

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CHAPTER 1

Introduction

Institutional transmission of tuberculosis (TB) is an important public health problem, especially in resource limited settings where protective measures are difficult to implement. A number of environmental controls exist to prevent nosocomial TB transmission, but many have not been fully evaluated due to difficulties in measuring TB transmission and therefore the effect of any intervention. Natural ventilation has been recommended as a control measure, but to date there is no evidence base for its efficacy for the prevention of tuberculosis transmission. The first part of this thesis is concerned with an investigation of the rates, determinants and effects of natural ventilation in health care facilities. The second part of this thesis deals with the creation and validation of a guinea pig air sampling facility for TB on the roof of a ward TB patients co-infected with HIV. This has given novel insights into tuberculosis transmission in the modern era of HIV infection and multidrug-resistant TB. Building on the experience gained in this PhD project, this guinea pig air sampling model in an improved animal facility is currently in use for the evaluation of low-cost, low-technology strategies to reduce airborne institutional TB transmission appropriate for low resource settings, where the burden of TB is highest.

1.1 Tuberculosis background

1.1.1 Global scale & the situation in Peru

Tuberculosis kills an estimated 1.8 million people worldwide each year and infects a further 8 million.[1,2] Whilst TB has re-emerged as a major public health problem in many developed countries, the main burden of this disease lies in low resource settings.[1] Peru has a high incidence of TB, reported to be 196 per 100,000.[3] The majority of cases are found in Lima, a city of over 8 million, many of whom live in overcrowded shanty town conditions. Since 1991 the National Tuberculosis Programme in Peru successfully operated a model DOTS (directly observed therapy, short course) treatment programme resulting in improvements in coverage and cure rates.[4] Despite this TB remains a major problem in Peru and there is a high prevalence of multidrug-resistant TB (MDR-TB).[5]

1.1.2 Institutional spread of tuberculosis

Nosocomial transmission of TB has been documented for many years,[6,7] but with the resurgence of TB in the 1980s and 1990s in the developed world numerous outbreaks occurred in European and north American hospitals.[8-12] Factors associated with these outbreaks include HIV infection, delayed diagnosis and MDR-TB,[8-10] which are associated with the prolonged respiratory dissemination of mycobacteria, and poor hospital infection control practices.[13] In recent years, large scale transmission of TB in homeless shelters,[14,15] residential facilities,[16,17] and prisons[18-20] has been documented using epidemiological evidence corroborated by DNA fingerprinting. Furthermore, TB

outbreaks are being increasingly recognised in other non-healthcare settings such as schools,[21,22] bars,[23,24] crack houses,[25] and church choirs.[26]

A number of studies in industrialised countries have demonstrated an increased incidence of tuberculosis infection amongst health care workers (HCWs) compared with the general population.[27-31] Increased risk of TB infection has also been shown in medical or nursing students with patient contact.[32,33] With a considerable proportion of TB patients being hospitalised or diagnosed in hospital settings,[34] nosocomial TB transmission and its control remains a priority in developed countries. However it is nosocomial transmission in low resource settings where controls are hardest to implement that is of particular importance.[35] Data concerning this problem were limited until a number of studies were conducted since the 1990s in a variety of low resource settings.[36-41] A recent study in Peru showed that physicians in a public hospital in Lima had a 17% annual tuberculin skin test conversion rate, compared with 3% in a local shanty town population.[42] A study in another Lima hospital demonstrated a high rate of active pulmonary TB among HCWs, 4-8 times higher than that in the general population of this high-burden country.[43] A recent Brazilian study demonstrated medical students to be at increased of TB infection in their later clinical years.[44]

In low resource settings hospital wards are often overcrowded, and cases of potentially highly infectious TB may often go undiagnosed. In one study in a general medical ward in Lima, 40% of patients had undiagnosed TB, of whom 15% had MDR-TB.[45] The high burden of HIV infection compounds the problem of nosocomial TB transmission in many low resource settings.[35] Nosocomial transmission to HCWs is especially important in developing countries, where HCWs are often a scarce but

valuable resource. Preventing tuberculosis in this group not only reduces overall TB burden, but reduces the possibility that an infected HCW spreads disease to a large number of susceptible individuals in their work environment. HCWs are integral to the success of DOTS programmes, and preventing TB in this group prevents their loss, either temporary or permanent, from health care systems. It is not only HCWs who are at risk from nosocomial transmission. Other patients sharing crowded wards are at increased risk, and in low resource settings patients' family members commonly spend long periods on wards providing basic care and food, and children often accompany their parents.

1.1.3 Multidrug-resistant TB

The prevalence of MDR-TB is increasing across the globe.[46,47] MDR-TB is associated with increased mortality,[48] especially in those co-infected with HIV.[49] Standard first line DOTS therapy for MDR-TB has poor cure rates, ranging between 5%[50] and 60%.[51] Treatment for MDR-TB requires prolonged therapy with multiple second-line agents, many of which have significant adverse effects which can make treatment more difficult.[52] Therapy for MDR-TB is expensive and is often unavailable in low resource settings, making untreated MDR-TB patients an important source of infection. Such patients are likely to cause ongoing transmission in their communities, and may contribute further to nosocomial transmission as they continue to seek attention in healthcare facilities.[35] In national TB control programs, empirical re-treatment regimens are sometimes based on the same four drugs as first-line therapy with the addition of streptomycin. If these regimens fail there may be an 'amplifier effect' on resistance, with strains becoming resistant to four or five drugs.[53,54] The lack of culture based diagnosis in many parts of the low resource world makes early

diagnosis and initiation of effective chemotherapy difficult for drug-resistant cases and as a result some are treated with sub-optimal regimens.

In Peru, MDR-TB is responsible for approximately 3% of previously untreated cases[47,55] but may be much higher. In one study of unselected female hospital in-patients, 13% had un-diagnosed TB, of whom 20% had MDR-TB.[45] Selected groups such as those failing DOTS have a higher prevalence of MDR-TB, and a national survey found that 15.7% of previously treated cases had MDR-TB.[55] A study in central Lima demonstrated 75% of treatment failures had MDR-TB[56] and another in northern Lima demonstrated an MDR-TB prevalence of 90% in patients failing first line therapy.[57] In addition MDR-TB strains in Lima are often highly resistant. The annual prevalence of isolates with resistance to at least five first-line drugs was 29% in one series, increasing to 37% by the end of the five year study period.[58]

1.1.4 HIV and tuberculosis

Infection with HIV results in increased susceptibility to TB infection even at relatively high CD4 counts.[59] There is a markedly increased risk of progression to TB disease, of approximately 10% per year,[60,61] and accelerated progression of TB disease itself.[16] Whilst highly active antiretroviral therapy results in major reductions in TB incidence amongst HIV infected patients, TB risk remains elevated.[62] TB infection up-regulates retroviral replication in vitro,[63] and co-infected TB-HIV patients have double the risk of death compared with patients infected with HIV alone, which appears to be due to accelerated HIV infection rather than TB.[64] The importance of exogenous re-infection is increasingly recognized in both HIV-negative and HIV-infected individuals.[65] Thus a high proportion of HIV patients become infected with TB, and the stigma that is

often attached to HIV infection has now become associated with tuberculosis, resulting in adverse effects on previously effective tuberculosis control programmes.[66]

1.2 Transmission of tuberculosis

1.2.1 Physics of airborne transmission

Following Koch's discovery of the tubercle bacillus in 1882, it was thought that transmission occurred by means of particles arising from dried sputum or contaminated articles such as bed linen or handkerchiefs. In 1897 it was Flugge who initially suggested the concept of small droplets being the vehicle for TB transmission, and he demonstrated infection of guinea pigs via inhalation of respiratory droplets from TB patients.[67] It was not until the 1930s that Wells developed the concept of the droplet nucleus and the real airborne nature of TB transmission with a series of elegant experiments.[68,69] Droplet nuclei are particles 1-5μm in diameter that result from the evaporation of larger droplets produced by respiratory manoeuvres such as coughing, sneezing or speaking. Duguid investigated the numbers of particles generated by such manoeuvres: speaking produced up to 210 particles; coughing up to 3,500; and sneezing 4,500 - 1 million.[70] Later work by Loudon showed that five minutes of loud talking produced the same number of infectious particles as a single cough.[71] Under standard conditions, due to its large surface area to volume ratio, a droplet of 100 μm diameter would take 1.3 seconds to evaporate, during which time it would fall only 0.42 m.[69] The settling tendency of droplet nuclei is thus negligible, such that these particles may remain suspended in the air for long periods, passively following air

currents.[72] When inhaled they are sufficiently small to by-pass the upper airway defences and reach the alveoli in order to establish new infection.

1.2.2 Airborne transmission to guinea pigs: the work of Riley

In an elegant series of experiments in the 1950s-60s,[73-76] Riley and his colleagues used guinea pigs exposed to exhaust air from a TB ward to demonstrate definitively the airborne nature of TB transmission by particles with the characteristics of droplet nuclei. Over four years, an average 125 guinea pigs were continuously exposed to air from a ward of six TB isolation rooms, resulting in 134 guinea pig infections. Drug susceptibility profiles and temporal exposure patterns implicated a human source of animal infection in most cases. These studies demonstrated a great variability in infectiousness of individual patients: of 130 patients who occupied the ward, just eight patients accounted for 46% of guinea pig infections.[77] In the second two years of the study, untreated patients were introduced into the ward, and markedly decreased infectiousness following commencement of effective treatment was demonstrated.[76] In addition, air disinfection by placement of UV lights in ducts was proven.[76] A re-creation of these classic studies and development of Riley's work using modern molecular tools in today's era of HIV infection and MDR-TB forms a central part of this thesis.

1.2.3 Infectiousness of patients

Riley's work demonstrated a great heterogeneity of infectiousness amongst TB patients.[77] The most powerful predictor of infectiousness is sputum smear status and there is ample epidemiological evidence from contact studies to demonstrate a clear

and consistent association between positive microscopy of sputum for TB bacilli and the prevalence of TB infection and disease amongst household contacts.[78,79,80] However, there exists considerable variation between these studies, with prevalence rates in contacts from 39% up to 65%.[80] Recent studies have demonstrated the importance of smear negative transmission, which was shown to account for approximately 17% of transmission in a study of 1,359 TB cases in San Francisco, USA.[81] Other important determinants of infectiousness have been demonstrated, including the presence of lung cavitation on chest X-ray and cough frequency,[82] and cough inducing procedures have been associated with extensive TB transmission.[7,30,83] It is likely there are multiple additional factors, including for example sputum volume and consistency,[84] as well as TB strain variables such as ability to become aerosolised and to survive in the airborne state withstanding desiccation and exposure to natural UV light, until deposition on the susceptible lung tissue of a new host.

HIV infection has an ill-defined effect on the infectiousness of TB patients, with studies of TB prevalence in household contacts of HIV positive compared with HIV negative TB patients showing conflicting results.[85] Reduced cavitation seen on chest X-ray and reduced bacillary load in sputum have been suggested as possible mechanisms of reduced infectivity.[86,87] Conversely, delayed diagnosis due to atypical presentations may result in increased transmission.

The effect of anti-tuberculous chemotherapy on reducing infectiousness is marked,[75,80] but the duration of patient infectiousness is less well defined.[88] A direct effect in reducing the numbers of mycobacteria in the lung is likely to be an important mechanism for reducing infectiousness. One study using sputum culture showed a reduction of 99% in the number of mycobacteria in the sputum of TB patients after an

average of 16 days of multiple anti-tuberculous drug therapy.[89] More recent work has better characterised the exponential killing of mycobacteria in the first two days of treatment, principally by isoniazid, followed a slower phase of bacterial killing.[90] Laboratory studies using mRNA as a marker have shown a dramatic reduction in mycobacterial viability in sputum in the first 48 hours following commencement of effective anti-tuberculous chemotherapy.[91] Controlled patient studies have shown that 85-90% of initially smear positive patients treated with recommended short-course chemotherapy regimens have negative sputum cultures at two months.[92,93,94] An additional postulated mechanism for the effect of chemotherapy on patient infectiousness involved the concept that low drug concentrations in respiratory secretions and saliva would be multiplied many times as the water in a respiratory droplet evaporated, impairing mycobacterial viability, though this was not demonstrated by the experiments of Loudon.[95] Effective anti-tuberculous chemotherapy further exerts its effect on patient infectiousness by reducing cough frequency, as shown by Loudon.[82]

In the United Kingdom, it is recommended that newly diagnosed patients are isolated for the first two weeks of their treatment after which they are generally considered non-infectious,[96] though the evidence base for this is slim.[88] In North America patients are required to produce three negative sputum smears on separate days and also to demonstrate clinical improvement with evidence of treatment adherence before this assumption can be made.[97] In a recent Canadian study of sputum smear positive patients with moderate to advanced disease, time for conversion to a culture negative sputum smear varied remarkably, between eight and 115 days.[98] In addition, in contrast to previous studies which demonstrated a high incidence (20-60%) of smear

positive but culture negative specimens in the first months of treatment,[93,99] the Canadian study, which used more sensitive liquid culture media than the solid media used in the older studies, found that 98% of such smear positive 'on-treatment' sputum specimens were in fact also culture positive.[98] Thus the determinants and duration of patient infectiousness, particularly in today's era of HIV infection and MDR-TB, remain incompletely understood.

1.2.4 Transmission of drug-sensitive *vs.* drug-resistant tuberculosis

The patients studied by Riley with drug-sensitive disease were four to eight times more likely to infect guinea pigs than those with drug-resistant disease, though there was great heterogeneity between patients.[76] Early work had shown isoniazid resistant strains of *M. tuberculosis* to grow less vigorously in guinea pigs,[100,101] however subsequent work demonstrated a wide variation in the virulence for guinea pigs of clinical TB isolates,[102,103] and that transmissibility as well as virulence appeared to be independent of drug resistance.[104] Three epidemiological studies of the contacts of both drug-resistant and drug-sensitive tuberculosis patients have shown no difference in relative transmissibility.[105,106,107] Three other studies, utilising a molecular epidemiological approach, showed reduced clustering (and therefore presumably reduced transmission) with drug-resistant disease,[108,109,110] whilst conflicting results were seen in a further study that demonstrated increased clustering.[111]

1.3 Prevention of airborne tuberculosis transmission

The probability of an exposed person acquiring TB by the airborne route is dependent on two main factors: the concentration of infectious droplet nuclei in the air and the duration of exposure. A variety of guidelines exist for the control of TB transmission in health care settings, perhaps the most authoritative being those produced by the Centers for Disease Control and Prevention (CDC) in Atlanta, USA.[112] The original 1994 guidelines were recently updated in 2005.[113] Whilst aimed at hospitals in the USA, these guidelines are often used for reference around the world. However guidelines need to be tailored to individual countries, which often have very different TB-related problems and resources with which to address them, which is particularly true in less developed countries.[114] The World Health Organisation has produced useful guidelines specifically aimed at low resource settings.[40]

The CDC guidelines have developed the concept of a hierarchy of control measures aimed at preventing the spread of TB in health care environments.[40,112,113] These priorities are administrative controls, environmental controls, and personal respiratory protection.

1.3.1 Administrative controls

Administrative controls are the highest priority and their aim is to achieve the diagnosis and isolation of TB patients and the initiation of effective treatment as rapidly as possible. Prompt and accurate TB diagnosis relies on well functioning laboratories able to perform sputum smear examinations. These are not always available in low resource settings, and with the diagnostic sensitivity of sputum smear

examination being only moderate,[115] administrative controls for TB transmission in health care facilities have limits to their success.

1.3.2 Environmental controls

Environmental controls are the second priority for the control of TB transmission in health care settings and are intended to reduce the airborne concentration of infectious droplet nuclei. At the simplest level this involves maximising natural ventilation by opening windows, and if possible controlling the direction of airflow such that air contaminated by coughing patients might flow out of a window rather than into another ward, or over a nursing station. With increased resources, mechanical ventilation systems that provide high rates of fresh air dilutional ventilation and directional airflow using negative pressure can be employed in high risk areas where tuberculosis patients are housed. As adjunctive measures, high efficiency particulate air (HEPA) filtration of re-circulated air or upper room ultraviolet germicidal irradiation (UVGI) may be used.[112,113]

1.3.3 Personal respiratory protection

The third priority for TB control in health care settings is the use of personal respiratory protection in high risk areas. High quality particulate respirators may provide good protection to the wearer, but their theoretical relative efficacy is limited for high intensity exposures and diminishes at high ventilation rates.[116] Particulate respirators must be tightly fitting to the face to be effective, but in practice there are often leaks around the facial seal which substantially reduce protection.[117] At approximately £1 each their expense precludes widespread use in many low resource

settings. If indeed they are provided for use by HCWs, a single disposable respirator is often required to be used for two weeks or more. As a result respirators frequently become deformed by improper storage in pockets or being hung for convenience around necks, and become increasingly ineffective as filter integrity is disrupted. Particulate respirators can only protect if they are actually worn, and they are often not worn in out-patient waiting rooms, emergency departments and general medical wards, the very places where highly infectious undiagnosed, untreated TB cases are likely to be found. In addition, staff adherence with hospital policies on respirator use is often poor, even in high risk settings.[118] In one study in Peru, only 7% of 95 physicians studied reported consistent respirator use when examining active TB cases.[42] Many other workers, such as cleaning and catering staff, enter areas housing infectious tuberculosis patients and rarely have access to particulate respirators. In Peru, as in many low resource settings, wards are often overcrowded, and family members and visitors spend long periods in these potentially infectious airspaces providing purchased medications and food for their sick relatives or friends. Environmental controls for TB transmission, which include dilutional ventilation, thus acquire additional importance in these low resource settings.

1.4 Ventilation

Institutional spread of tuberculosis is most likely in conditions of overcrowding and poor ventilation. Inadequate air exchange rates or failings in mechanical ventilation systems have been implicated in numerous outbreaks.[6,7,12,83,119,120] Poor household ventilation has been associated with increased TB infection in household contacts[121] and tuberculin skin test conversion in hospital HCWs has been strongly associated

with inadequate ventilation in general patient rooms.[122] Whilst this epidemiological evidence for the effect of ventilation on nosocomial TB transmission is based on the observation that poor ventilation appears to be a contributory factor in outbreaks, there are clear theoretical reasons to suggest why increased dilutional ventilation should reduce the risk of airborne disease transmission.[123]

1.4.1 Natural ventilation

Natural ventilation offers the simplest means for reducing the airborne concentration of droplet nuclei and is most applicable in tropical or temperate climates. Ventilation may be maximised by keeping windows and doors open, with room designs that facilitate the inflow of clean air on one side of a room, and the exhaust of contaminated air on the other.[40] Old-fashioned sanatoria style hospitals with high ceilings and large open windows were designed with good natural ventilation in mind, in part because fresh air 'therapy' was prescribed for TB patients in the pre-antibiotic era.[124] Reduced nosocomial TB transmission is likely to be facilitated by careful planning in the positioning outside in the open air of out-patient waiting room queues, for example, which may contain highly infectious undiagnosed TB patients. Whilst natural ventilation is recommended by the World Health Organisation for the control of TB transmission in low resource settings,[40] to date there have been no published studies of the efficacy of natural ventilation for the prevention of institutional TB transmission.

1.4.2 Mechanical ventilation

Mechanical ventilation systems for the control of airborne tuberculosis transmission are designed to deliver high volumes of fresh air in order to dilute the concentration of infectious droplet nuclei, and to exhaust infected air out to the environment, sometimes after filtration. By exhausting approximately 10% more air than is supplied, a negative pressure is generated which minimises the escape of droplet nuclei from an isolation room when the door is opened. This is further enhanced by the use of anterooms between corridors and respiratory isolation rooms. One air-change/hour is defined as one room volume of fresh air entering that room in one hour, whilst an equal volume is exhausted. Assuming perfect mixing, 37% of old air will theoretically remain after one hour, and the mathematical function that describes this effect is a geometric one, such that by doubling the ventilation rate the particle residence time in the room space is halved. The theoretical effect of increasing air-changes/hour on the concentration of particles in the air in a room is shown in Figure 1.1. It can be seen that at one air-change/hour 276 minutes are required for an airborne particle removal efficiency of 99%, which reduces to 46 minutes at six air-changes/hour and 23 minutes at 12 air-changes/hour. By applying the Wells-Riley model of airborne infection[123] (see section 1.5.1) to different infection scenarios with different levels of ventilation, Nardell has shown that the probability of infection is reduced as ventilation increases.[125] However, the benefits of increasing ventilation become progressively smaller at higher rates of ventilation, and there are limits to the protection that can be achieved from dilutional ventilation. Furthermore, the relative protective effect of ventilation decreases as the infectiousness of the source increases (for example during a bronchoscopy).[125] For engineering reasons, it becomes impractical to increase mechanically delivered air exchange rates above certain levels

due to cost, vibrations from ductwork and extractor fans, noise, and drafts from air supply vents affecting patients.

It should be noted, as Beggs and colleagues have pointed out,[126] that the number of susceptibles who become infected in models of airborne infection such as the Wells-Riley model is influenced by absolute ventilation per person rather than air-changes/hour, on which many guidelines are based. Thus the use of air-changes/hour alone to denote ventilatory protection from infection may sometimes be deceptive. Air-changes/hour (ACH) are relative measures of ventilation influenced by room volume: ACH = absolute room ventilation (m^3/h) divided by room volume (m^3). For example, an isolation room with a floor area of 12 m^2 and a ceiling three meters high ventilated at 12 ACH provides 432 m^3/h of absolute ventilation per person (12 m^2 x 3 m x 12 ACH = 432 m^3/h). A room with the same floor area but with a four meter ceiling ventilated at only 9 ACH provides the same 432 m^3/h of absolute ventilation per person (12 m^2 x 4 m x 9 ACH = 432 m^3/h). The theoretical protection against airborne infection provided by ventilation would be identical in each room, even though the second room has fewer air-changes/hour. There may also be a protective benefit from increased dilution of infectious particles in larger room volumes, an effect not appreciated by modelling using the Wells-Riley equation as it assumes perfect air mixing and steady state conditions.

Mechanical ventilation systems that provide high rates of dilutional ventilation and negative pressure are inappropriate in the majority of the less developed world, where the burden of TB is highest. Such facilities cost between tens and hundreds of thousands of pounds to install and more importantly they demand ongoing maintenance, which requires specific expertise as well as significant ongoing financial

commitment.[127] Filters must be changed regularly, fan blades cleaned, and air injection and extractor fan motors properly maintained. Regular monitoring of the direction of airflow into negatively pressurised rooms must be carried out, as if the air extraction system is underperforming compared with the air supply system, perhaps due to a saturated filter, positive pressure in an isolation room will result. Indeed, many TB outbreaks in the developed world have been associated with inadequate air exchange rates,[7,119] incorrect direction of airflow,[8,6,12] or re-circulation of infected air.[6,12,16,128] A survey conducted in 1992 of 729 US hospitals found that 29% did not have any respiratory isolation rooms which met recommended standards.[129] A survey of 17 Canadian hospitals showed only 24% of respiratory isolation rooms had both adequate air exchange rates and direction of air flow,[122] and in a US study 45% of 115 negative pressure isolation rooms actually had positive pressure.[130] A study of 140 respiratory isolation rooms in 38 facilities in New York State, USA, demonstrated inappropriate outward flow of air (i.e. positive pressure instead of negative pressure) in 38%, and a further study found that continuous monitoring devices for negative pressure had poor reliability, conflicting frequently with simple smoke tests for demonstrating direction of airflow.[131,132] Maintenance of such mechanical ventilation systems is thus crucial, but such maintenance is likely to be more difficult in low resource settings.

1.4.3 Measurement of ventilation

Measurement of ventilation in isolation rooms with mechanical ventilation systems can be performed by measuring airflow in ducts or at air injection and exhaust vents using equipment such as vane anemometers, thermal anemometers or balometer capture hoods. More accurate measurements can be made by using tracer gas techniques,[133,134] but these techniques require special tracer gases and sophisticated

gas detection systems. This may be avoided by using carbon dioxide as a tracer gas which can be easily detected using an inexpensive infra-red gas analyser, as described in a simple protocol devised and validated by Menzies and co-workers.[135] In naturally ventilated rooms, measuring air flow at windows and doors to assess total room ventilation is inaccurate owing to the inherent variability in natural air currents and therefore a tracer gas technique is the most appropriate methodology.

1.5 Modelling airborne TB infection

The infectious dose of TB for humans is not known. For this reason William Wells developed the concept of a 'quantum of infection' as the infectious dose, whether this is one or several organisms.[84] He also pointed out the Poisson relation between the number of infectious particles breathed by susceptibles as a population and the number who become infected, such that under conditions where each susceptible breathed in one quantum of infection on average, 63% ($1\text{-}e^{-1} = 0.63$) of susceptibles would become infected. This arises because when particles are randomly distributed in air, some susceptibles would inhale more than one infectious particle, whilst others would inhale none at all and thus escape infection.

The concept of infectious quanta has allowed the development of mathematical models to study airborne TB transmission. Several have been used, including the Mass Action Model,[72] the Wells-Riley model,[123] and a more complex model developed by Gammaitoni and Nucci.[136] All these models have limitations, in part through the modelling assumptions that must be made.[126] These include the assumption that all susceptibles have an identical pulmonary ventilation rate, that the air in a room space

is completely mixed, and that all susceptibles are equally vulnerable to the infectious agent. In addition, these models do not easily allow for infected susceptibles becoming infectors themselves, though in the case of TB the relatively long pre-infective incubation time makes this limitation less important. The Wells-Riley model described in the next chapter is relatively straightforward and has been used by Riley to estimate the infectiousness of the TB patients on the ward beneath his guinea pig air sampling facility.[123] This model was also used by Nardell to describe a TB outbreak in an office building and to determine the theoretical limitations of protection achievable by ventilation.[125] The Wells-Riley model has therefore been chosen for use in this thesis, first to model the effect of natural ventilation on TB transmission, and later to describe the infectiousness of HIV-TB co-infected patients.

1.6 Conclusion

Tuberculosis remains a major global public health problem, and the HIV-AIDS pandemic has had a major adverse impact on efforts to control this ancient disease. There remain many unanswered questions concerning the aerobiology of this disease, and much of what we know about airborne tuberculosis transmission is based on experiments conducted almost 50 years ago. Whilst determinants of patient infectiousness such as sputum smear status and cough frequency are well documented, relatively little is known about why some patients are tuberculosis disseminators. Nosocomial and other institutional transmission of tuberculosis remains an important problem. Whilst control measures in the developed world appear to have been relatively successful, reducing institutional spread of tuberculosis represents a significant challenge in low resource settings. There is an urgent need for low-cost,

low-technology control strategies, and this has prompted the work on which this thesis is based.

This thesis is concerned with the detection and prevention of airborne tuberculosis transmission. The following chapter describes an investigation of the rates and determinants of natural ventilation by simply opening windows in a wide variety of hospital settings. Mathematical modelling is used to evaluate the efficacy of this natural ventilation for the prevention of institutional TB transmission. However, natural ventilation is not appropriate in cold climates, for example parts of Russia and Eastern Europe where the prevalence of TB and MDR-TB is high.[46] Thus other environmental controls for the prevention of airborne TB in healthcare settings are required. To enable the evaluation of strategies to prevent airborne TB transmission, a reliable method for detecting TB transmission is needed. Conventional mechanical air sampling for TB is made difficult by the paucity of airborne organisms,[76] damage through the physical stresses of sampling, and fungal overgrowth of samples.[137] Newly developed air sampling techniques based on nucleic acid detection are unable to distinguish between living and dead organisms.[138] Thus for the first time in almost 50 years Riley's classic guinea pig model to detect airborne TB has been successfully re-created, in Lima, Peru. The optimisation of this air sampling model is described in chapter three. By using modern molecular tools to determine which patient was responsible for which guinea pig infection, this model has allowed new insights into the infectiousness of tuberculosis patients in today's era of HIV infection which are presented in chapter four. Upper room ultraviolet light is recommended as an adjunct for the control of TB transmission in healthcare settings, but there are no controlled field studies of its efficacy.[112] The negative ionisation of air offers promising potential

to interrupt airborne disease transmission,[139-141] but there are currently no studies on the effect of negative air ionisation on TB transmission. Both of these relatively low-cost, low-technology interventions are currently being evaluated using the guinea pig air sampling facility developed through the work described in this thesis.

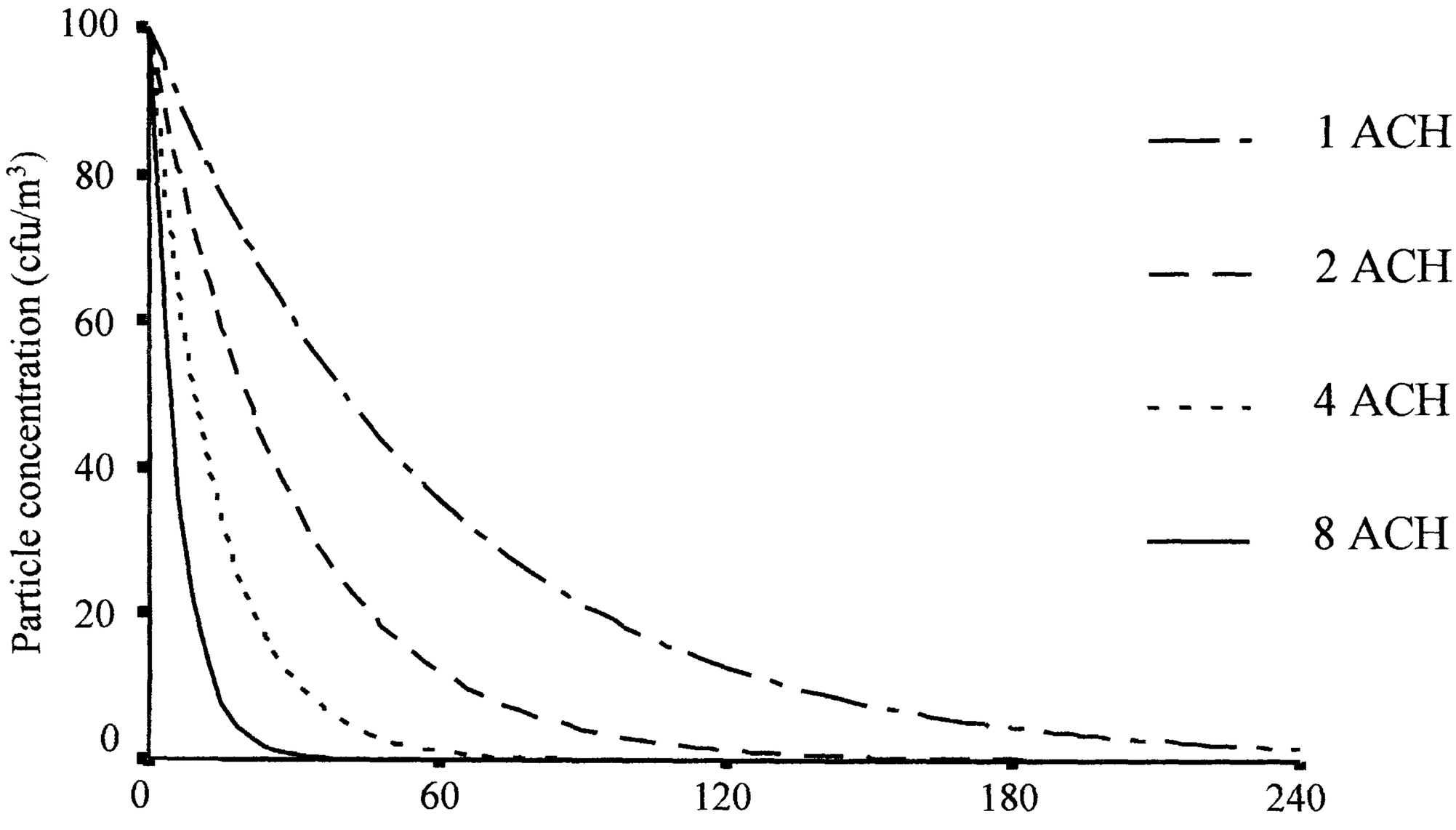


Figure 1.1: Effect of increasing air-changes/hour (ACH) on concentration of infectious particles in a room. The theoretical reduction in the concentration of infectious particles, initially 100 colony forming units (cfu)/m^3, is shown for a room with dilutional exhaust ventilation at 1 ACH, 2 ACH, 4 ACH and 8 ACH. Perfect mixing of room air is assumed. The decreasing benefits of increasing ACH at high levels of ACH can be observed. This graph is derived from the formula for the rate of purging airborne contaminants.[112,142]

CHAPTER 2

Natural ventilation to prevent airborne tuberculosis transmission

2.1 Introduction

Ventilation protects against tuberculosis and other airborne infections by diluting infectious particles with uncontaminated air and removing infectious particles before they can be inhaled by a susceptible. Dilutional ventilation may be provided by mechanical ventilation systems, but these are expensive to install and require ongoing maintenance and expertise. The simplest means of ventilating a room is by opening windows and doors, known as natural ventilation. Natural ventilation is particularly applicable in temperate or tropical climates, where coincidentally much of the global burden of tuberculosis is found. Indeed, natural ventilation is recommended by the World Health Organisation as a TB control measure in resource-limited settings.[40] However there have been no studies of the rates of air exchange achievable through natural ventilation, the determinants of natural ventilation, or its efficacy for reducing airborne tuberculosis transmission.

In this study natural ventilation was measured in a wide variety of clinical settings where tuberculosis patients may be found in hospitals in Lima, Peru. Environmental and architectural determinants of natural ventilation were investigated, with particular interest in old-fashioned clinical facilities built before 1950, which generally had high

ceilings and large windows. The results were used to model the impact of natural ventilation on airborne tuberculosis transmission using a range of infectious source cases from the literature, compared with the protection provided by mechanical ventilation.

2.2 Methods

2.2.1 Setting

70 naturally ventilated rooms in 8 hospitals in Lima Peru were studied. Rooms were selected on the basis of the likelihood of them accommodating infectious patients, in particular tuberculosis patients. These included respiratory isolation rooms (n=13), wards for tuberculosis patients (n=13), respiratory wards (n=9), general medical wards (n=8), emergency departments (n=8), out-patient consulting rooms (n=6), tuberculosis control program clinics (n=5), wards for HIV and infection patients (n=4), nebuliser rooms (n=2) an autopsy room (n=1), and a respiratory out-patients waiting room.

12 mechanically ventilated rooms were studied in Hospital Nacional Dos de Mayo, Lima, Peru. This mechanically ventilated negative-pressure facility for TB-HIV infected patients was built in the year 2000, and is the only one of its kind in Peru. As part of this research, this facility was modified in 2003. The two 4-bedded wards were converted into eight isolation rooms and the mechanical ventilation system renovated to deliver increased air changes per hour in accordance with guidelines.

Ethical approval was obtained from the IRB of Asociación Benéfica PRISMA, Lima, Peru. Patient management was not affected by experimental protocols.

2.2.2 Tracer gas technique

406 experiments were performed using a tracer gas concentration-decay technique that quantifies the rate of carbon dioxide (CO_2) removal from a room by ventilation. 368 experiments were performed in naturally ventilated rooms and 38 in mechanically ventilated rooms. In the naturally ventilated facilities, windows and doors were closed, and all remaining apertures in the room, for example missing windows panes, were sealed as best possible using sheets or cardboard and adhesive tape. CO_2 was released from a cylinder or from 6 kg fire extinguishers to a peak concentration 3000-10,000 parts per million (ppm) depending on the size of the room. CO_2 was thoroughly mixed with room air using two large table fans. CO_2 concentrations were measured at 1-minute intervals throughout using an infra-red gas analyser (Gas Data Ltd, Coventry, UK) located centrally in the room. Windows and doors were kept closed for a period of 5-15 minutes (depending on room size and 'leakiness') following which they were opened, either sequentially or all at once. CO_2 concentrations were allowed to fall to baseline levels before experiments were repeated. The duration of each experiment was between 10 and 40 minutes, being shortest in well ventilated rooms. The experimental procedure was similar in mechanically ventilated rooms except that all windows and doors were always closed by definition, and there was therefore just one CO_2 concentration-decay curve indicating the rate of mechanical ventilation.

Measurements of ventilation were made at least three times in each room. In some rooms that were easily accessible to the study, measurements were conducted on multiple occasions at different times of the day under a variety of weather conditions and during different seasons of the year.

2.2.3 Calculation of air-changes/hour

Air-changes/hour for each experiment were calculated as the gradient of the straight-line through the natural logarithm of CO_2 concentration (ppm) plotted against time (hours).[135] Measurements were considered from peak CO_2 concentration after air mixing with the fans, generally between 3000-10,000 ppm depending on room size, until CO_2 concentration fell to within 200 ppm of baseline to allow for CO_2 production by room occupants. For each experiment, ventilation measured in air-changes/hour was calculated for the period during which windows and doors were fully closed, and for the period during which windows and doors were fully opened. In experiments where windows and/or doors were opened sequentially, a calculation of air-changes/hour was also done for the partially open state – i.e. just some of the total numbers of windows and doors open.

2.2.4 Architectural determinants of natural ventilation

For each room, its type and number of occupants (both actual and maximum) was recorded, and the following architectural information was noted: floor area (m^2), ceiling height (m), total area of widows and doors (m^2), area of windows and doors open during each part of the experiment (m^2), floor elevation from ground (m), exposure of room to prevailing winds, and the presence of open windows or doors on opposite walls to allow unobstructed flow of air through a room. Exposure of room to prevailing winds was measured as the incident angle of prevailing wind to the room's main open windows. In Lima, a coastal city, prevailing wind comes principally from the Pacific Ocean.

2.2.5 Environmental determinants of natural ventilation

For each experiment, the following environmental information was recorded: temperature (^{0}C) relative humidity (%) and season. Lima has only two easily discernable seasons. Winter was defined as May to October inclusive. Summer was defined as November to April inclusive. For each measurement of ventilation, wind speed was recorded at the window using a thermal anemometer (TA 35 Airflow Technical Products Inc., Andover, USA).

2.2.6 Modelling of airborne tuberculosis transmission

The Wells-Riley equation[123] was used to model the risk of airborne TB transmission:

$$C = S(1-e^{-Iqpt/Q})$$

where: C = number of new cases

S = number of susceptibles exposed

I = number of infectors

q = infectious quanta produced per hour

p = pulmonary ventilation (m^3/hour)

t = exposure time (hours)

Q = absolute ventilation of room (m^3/hour)

Four different scenarios were modelled, using q values from published literature for four different infectious tuberculosis sources.

- q=1.3: standard TB patients from Riley's four year study of the infectiousness of a heterogeneous group of TB patients, using guinea pigs on the roof of a TB ward.[75,76,123] A mixture of treated and untreated patients was studied by Riley, including those with both drug-resistant and drug-sensitive TB strains.

- q=13: an office worker with untreated pulmonary tuberculosis reported by Nardell and colleagues, who infected 27 office co-workers over a four week period before diagnosis.[125]

- q=60: the most infectious case studied by Riley, a patient with laryngeal TB who infected 15 guinea pigs during a three day stay on the unit.[76]

- q=250: an outbreak related to a bronchoscopy procedure on a TB patient.[7,125]

For the first three infectious sources, infection risk was modelled using a 24 hour exposure period. For the fourth scenario, the bronchoscopy, an exposure of one hour duration was utilised. One hour instead of 24 hours was selected for the bronchoscopy case because for such an infectious source at 24 hours the majority of susceptibles would become infected, such that the differences in protection afforded by ventilation in the different categories of room would not be appreciated. In addition, the duration of a real life exposure to a bronchoscopy is likely to be close to one hour. Susceptibles were assumed not to be protected by particulate respirators. To allow comparison between isolation and multiply occupied rooms, all patients in each room were assumed to be tuberculous infectors of equal infectiousness, and the total absolute

ventilation for each room, Q (m^3/h), was used. This is equivalent to considering the number of infectors, I, equal to one, but using 'Q per person' (m^3/h/person) as the value for room ventilation. A standard value of 0.6 m^3/hour for pulmonary ventilation has been used.[125] The calculated risk of infection is expressed as the percent of susceptibles that would become infected.

2.2.7 Statistical Analysis

Numerical data was entered into Excel spreadsheet computer software (Office version 2003, Microsoft Corporation) which was also used for plotting simple graphs. For statistical analyses including Spearman correlations, Mann-Whitney U tests, univariate and multiple linear regressions, and the more complex graphs, these Excel spreadsheets were directly transferred into SPSS version 10, SPSS Inc., Chicago, USA, or Stata version 8.0, Statacorp LP, Texas, USA, if data were clustered. Non-parametric tests were used unless histograms demonstrated that the data fitted a normal distribution. Determinants of ventilation and transmission risk were first assessed by univariate analysis. Associations with $p<0.15$ were included in a linear regression model. In all regressions dependent variables were normalised by $\log_{10}$-transformation and allowed for clustering by room. In the 'box-and-whisker plot' graphs the boxes represent the inter-quartile ranges around the median and the whiskers are drawn between the lowest and highest values after excluding outliers. Outliers are defined as beyond 1.5 x the inter-quartile range above the 75th or below the 25th percentile.[143] In the text medians and inter-quartile ranges of all the data are used unless stated. Statistical significance was defined as $p\leq0.05$.

2.3 Results

2.3.1 Carbon dioxide concentration-decay experiments

Figure 2.1 shows a typical carbon dioxide concentration-decay graph, in this case for an experiment conducted in a respiratory ward. Baseline CO_2 concentration was 500 parts per million (ppm). CO_2 was released after one minute, and CO_2 concentration increased rapidly to a peak of 5950 ppm at time 3 minutes. Large table fans were used to mix room air for the first five minutes. A second value of 5950 ppm was observed at time 6 minutes. For the following 16 minutes, windows and doors were kept closed, and CO_2 concentration decayed slowly. At time 22 minutes, windows and doors were opened simultaneously. CO_2 concentration-decay then occurred much more rapidly, with levels returning to baseline.

2.3.2 Calculation of air-changes/hour

Figure 2.2 shows a graph used for the calculation of air-changes/hour for the period with windows and doors closed in the typical experiment just described. The graph shows the natural logarithm of CO_2 concentration plotted against time (in hours) from the peak CO_2 concentration after mixing (5950 ppm at 6 minutes) to the point when windows and doors were opened (5050 ppm at time 22 minutes). A straight line of best-fit has been drawn through the points on the graph. Air-changes/hour were calculated as the positive value for the gradient of this straight line, in this case 0.5 ACH. Figure 2.3 shows a similar graph but for the period from the point when windows and doors were opened (5050 ppm at 22 minutes) to the point where CO_2 concentration returned to within 200 ppm of baseline (700 ppm at 31 minutes). 12

ACH were calculated for this period, corresponding to natural ventilation with windows and doors fully open.

2.3.3 Effect of opening windows and doors on natural ventilation

Opening windows and doors resulted in increased rates of ventilation. With windows and doors fully open, median air-changes/hour were 28 (inter-quartile range 18-46), more than eighteen times the median 1.5 ACH (inter-quartile range 0.7-2.7) with windows and doors fully closed ($p<0.001$). The corresponding values for absolute ventilation were 2441 m^3/h (1154-4219 m^3/h) *vs.* 113 m^3/h (49-276 m^3/h) ($p<0.001$).

Opening an increasing number windows and/or doors in any one room was associated with increasing ventilation in that room. Figure 2.4 demonstrates a clear increase in ventilation measured in air-changes/hour as increasing numbers of windows and doors were opened in two tuberculosis wards, and one respiratory isolation room. Each 'box-and-whisker' of measured air-changes/hour represents one configuration of opened windows and doors, expressed on the non-linear x-axis as a percentage of the total room window and door area for that room.

Figure 2.5 demonstrates the effect of opening windows and doors on ventilation expressed as both air-changes/hour and absolute ventilation (m^3/h) for all data in naturally ventilated rooms. 'Closed' was defined as the state with windows and doors completely closed. In six rooms, some small apertures remained open as it was not possible to close them (for example missing panes in an inaccessible window). These apertures amounted to less than 15% of the total window/door area, and these rooms were included in the analyses of 'Closed'. 'Fully open' was defined as all windows

and doors as fully open as possible. In 28 experiments, all in three rooms, some windows became jammed shut during series of experiments, but the area fully opened was always >85% of total area and these experiments were included in analyses of 'Fully open.' In six rooms, it was not possible to measure ventilation with windows and/or doors fully open, as ≥15% of the total area was permanently shut. This was generally due to painting, or broken hinges that had resulted in windows being nailed shut. These six rooms are included in 'Closed' and 'Partially open' analyses only. 'Partially open' was defined as having at least one, but not all, of windows and/or doors open – i.e. anything not 'Closed' or 'Fully open'.

2.3.4 Effect of wind speed on natural ventilation

Median wind speed measured at the window was 2.8 km/h (inter-quartile range 1.9-36 km/h). Wind speed measured at the window was strongly correlated with incident angle of open windows to prevailing winds (Spearman co-efficient 0.57; $p<0.001$), and less strongly correlated with room height above the ground (Spearman co-efficient 0.15; $p=0.006$).

The effect of wind speed on natural ventilation is demonstrated in Figure 2.6. Natural ventilation measured in ACH increased with increasing wind speeds, which are divided in the graph into four quartiles. In naturally ventilated wards with fully open windows, even the lowest quartile of wind speeds resulted in greater ventilation than the 12 ACH recommended by guidelines for mechanical ventilated facilities ($p<0.001$).

2.3.5 Comparison of natural ventilation in old-fashioned pre-1950 *vs.* modern facilities, compared with mechanical ventilation

Five of the hospitals where ventilation measurements were carried out were built before 1950. The oldest hospital was built in 1875. Three of these old hospitals had modern emergency departments, built after 1970. The remaining three hospitals were built in the 1970s and 1980s. Rooms in the old hospitals were classified as 'pre-1950', except for their more recently built emergency department rooms. These emergency rooms were classified as 'modern', as were all rooms in the more modern hospitals. In total 27 rooms were classed as pre-1950, and 43 were classed as modern.

Compared with modern naturally ventilated rooms, pre-1950 rooms had larger volumes (85 *vs.* 60 m^3), higher ceilings (4.2 *vs.* 3.0 m), more windows and doors (total window and door area 6.6 *vs.* 3.4 m^2; total window and door area to room volume ratio 0.1 *vs.* 0.05), and were more likely to have windows on opposite walls to facilitate through-flow of air (56% *vs.* 19% of rooms) (all median values; all $p<0.05$). Pre-1950s rooms were orientated with greater exposure to prevailing wind but this difference was not significant (85^0 *vs.* 120^0 angle of incidence of main windows to prevailing wind; $p=0.9$). There was no significant difference in wind speed measured at the window in pre-1950 compared with modern naturally ventilated rooms (median 2.8 km/h *vs.* 2.8 km/h; $p=0.7$). Importantly for the calculations of infection risk, there was no significant difference in floor area per patient between pre-1950 and modern wards (median 9.2 *vs.* 9.3 m^2 floor-area/patient; $p=0.5$).

With windows and doors fully open pre-1950 facilities had substantially greater ventilation than their modern counterparts measured both as air-changes/hour and

absolute ventilation: 40 *vs.* 17 ACH and 3,769 *vs.* 1,174 m^3/h, respectively (both $p<0.0001$). Even these modern facilities, however, had greater ventilation than that calculated for the 12 mechanically ventilated respiratory isolation rooms in this study when ventilated optimally at 12 ACH according to guidelines[112,113]: 12 ACH and 402 m^3/h for absolute ventilation (both $p<0.0001$). These differences are also seen in absolute ventilation per person, derived from dividing total room absolute ventilation by the maximum number of patient beds for that room. Median absolute ventilation per person was 1557 m^3/h for pre-1950 rooms *vs.* 461 m^3/h for modern naturally ventilated rooms *vs.* 374 m^3/h for respiratory isolation rooms ventilated mechanically at 12 ACH (all $p<0.0001$ except for modern naturally ventilated facilities *vs.* mechanical ventilation where $p=0.02$). Figure 2.7 demonstrates the difference in ventilation between pre-1950 and modern naturally ventilated rooms and mechanically ventilated rooms for air-changes/hour, absolute ventilation, and absolute ventilation per person. Mechanically ventilated respiratory isolation rooms are shown as being optimally ventilated at 12 ACH according to guidelines[112,113] (see following section).

2.3.6 Mechanically ventilated facilities

The only negative pressure mechanically ventilated facility for patients with HIV-TB co-infection in Peru, located in Lima at Hospital Nacional Dos de Mayo, was included in this study. Created in the year 2000 by the division of a large open-style 1940s TB ward and installation of a false ceiling, it initially comprised two 4-bedded wards, one 6-bedded ward, and an isolation room. The mechanical ventilation system was designed to deliver negative pressure and 6 ACH.

The mechanically ventilated facility was found to deliver less than half the recommended air-changes/hour. On inspection, air supply and extraction fans were not protected by any filters, and fan blades were found to be badly corroded and clogged with deposits as shown in the photographs in Figures 2.8. As part of this research effort, the mechanically ventilated facility was further modified, and the ventilation system renovated with new ductwork, motors and fans, to allow it to deliver 12 ACH, as recommended by guidelines.[112,113] The two 4-bedded rooms were converted into eight isolation rooms and the remaining rooms were left unchanged except for improvements to the ventilation system. To improve the external validity of the comparison of ventilation and tuberculosis transmission risk in natural *vs.* mechanically ventilated rooms, the measured values of ventilation for the poorly maintained ward were not used. Instead, values for these 12 rooms were based on the 12 ACH recommended by guidelines.[112,113] Absolute ventilation (m^3/h) for these rooms was calculated accordingly by multiplying ACH (12) by room volume (m^3), and these calculated values are presented for mechanical ventilation in the text, figures and models of airborne infection. For these 12 rooms, ceiling height was 2.9 m, and median floor area per patient was 11 m^2 (9.4-12 m^2). Although floor area per patient tended to be greater in modern mechanically ventilated isolation rooms than naturally ventilated rooms (median 9.2 m^2 floor area per patient), this difference was not significant ($p=0.1$). As noted above in section 2.3.5, median absolute ventilation was calculated to be 402 m^3/h and median absolute ventilation per person 374 m^3/h.

2.3.7 Determinants of natural ventilation

Putative determinants of ventilation and tuberculosis infection risk (see next section) were first assessed by univariate regression. Associations with $p<0.15$ with at least

one of air-changes/hour or absolute ventilation or infection risk were included in a multiple linear regression model and can be seen in Table 2.1. All regressions were clustered by room and dependent variables normalised by log_{10}-transformation. Exposure of the room to prevailing winds was not included in these analyses because measured wind speed was already included, and being measured at the window, the value for wind speed recorded was in reality a composite of prevailing wind speed and the exposure of the window to prevailing wind. Temperature, relative humidity and season were not significant in univariate regression analyses and hence are not included in Table 2.1.

2.3.8 Calculated risk of airborne tuberculosis transmission

The median risk of tuberculosis transmission from 24 hours shared in a room with untreated tuberculosis patients (producing 13 infectious quanta per hour) varied from 97% for all naturally ventilated facilities with all windows and doors completely closed, to 39% in the mechanically ventilated respiratory isolation rooms ventilated at the recommended 12 ACH, to 33% in modern naturally ventilated rooms with windows and doors fully open, compared with 11% in naturally ventilated facilities built before 1950 with their windows and doors fully open. These calculated risks of infection are for susceptibles assumed to be unprotected by the use of particulate respirators.

Figures 2.9 and 2.10 demonstrate the estimated risk of TB infection for the four scenarios modelled, for pre-1950 and modern naturally ventilated facilities *vs.* mechanically ventilated respiratory isolation rooms ventilated at the recommended 12 ACH.[112,113] Whilst important variables in the Wells-Riley equation include 'q', the

rate of infectious quanta produced per hour by the source and 'Q', the absolute ventilation (m^3/h), so too is duration of exposure. With sufficiently long exposure, according to the model 100% of exposed susceptibles will become infected, irrespective of ventilation or the infectiousness of source patients. Figure 2.11 demonstrates the effect of duration of exposure on the theoretical risk of infection for three values of source infectiousness (q=1.3, 13 and 60) in three different ventilation scenarios: pre-1950 naturally ventilated rooms; post-1970 modern naturally ventilated rooms; and the year 2000 built mechanically ventilated respiratory isolation rooms ventilated at the recommended 12 air-changes/hour.[112,113] The median of all data for absolute ventilation (m^3/h) (with windows and doors fully open in the case of natural ventilation) for each category of room was used in the model.

2.4 Discussion

The results presented in this chapter demonstrate that natural ventilation resulting from simply opening windows and doors can provide high rates of fresh air exchange in a wide range of clinical settings. Even the lowest quartile of wind speeds resulted in higher ventilation than that found in mechanically ventilated facilities functioning optimally according to guidelines. Old-fashioned pre-1950 naturally ventilated wards were characterised by high ceilings and large windows and had the highest rates of ventilation. Calculation of the theoretical risk of tuberculosis transmission from a range of TB sources of different infectivity demonstrated that the risk of airborne infection was highest in mechanically ventilated rooms, and significantly lower in naturally ventilated rooms, being lowest in old-fashioned pre-1950 facilities.

2.4.1 High rates of ventilation achievable by natural ventilation, even at low wind speeds

Naturally ventilated facilities had a median absolute ventilation of 2477 m^3/h, more than six times the rate in mechanically ventilated respiratory isolation rooms ventilated according to guidelines, and more than twenty times that with windows and doors completely closed. Natural ventilation provided a median 28 air-changes/hour, more than double the 6-12 air-changes/hour currently recommended for the control of tuberculosis transmission in healthcare facilities.[112,113] High rates of air exchange were even maintained at low wind speeds. For experiments when wind speed was within the lowest quartile of wind speeds, for all naturally ventilated rooms with windows and doors fully open, median absolute ventilation was 1587 m^3/h and median air-changes/hour were 20, compared with 402 m^3/h and 12 ACH respectively for mechanically ventilated facilities ventilated optimally according to guidelines.

2.4.2 Old-fashioned rooms built pre-1950 with high ceilings and large windows provided highest rates of ventilation

Naturally ventilated rooms built before 1950 had significantly higher ventilation than their modern counterparts. Pre-1950 rooms had median 3,769 m^3/h absolute ventilation and median 40 air-changes/hour, compared with 1,174 m^3/h for and 17 air-changes/hour for modern naturally ventilated rooms, built post-1970. This striking difference in rates of ventilation between old-fashioned and modern facilities is explained by several factors. Of the environmental and architectural variables significantly associated in multiple regression analysis with measures of ventilation, pre-1950 facilities scored higher in all except for floor area, for which there was no

significant difference. Pre-1950 facilities had higher ceilings, and this in turn resulted in larger volume rooms on average. Pre-1950 rooms had greater total areas of windows and doors, and a greater ratio of area of windows and doors compared to room volume. This greater proportion of windows was in part responsible for pre-1950 facilities having greater potential for through-flow of air. 56% of pre-1950 rooms had windows on opposite walls to facilitate air through-flow, compared with just 19% of modern post-1970 rooms. There was no significant difference in wind speeds measured at the window in pre-1950 rooms compared with modern naturally ventilated rooms, and visits to wards were not organised according to weather reports. Pre-1950 rooms had generally greater exposure to prevailing winds but this difference was not significant ($p=0.9$).

For a room of a given dimensions, it is these factors (window and door area, air through-flow, and wind speed) that largely explain the finding that there was more than twice the amount of air-changes/hour in pre-1950 compared with post-1970 naturally ventilated rooms. However, the difference in absolute ventilation was even greater, being over three times as much in old-fashioned compared with modern facilities. This additional difference is explained by the larger median volume of the old-fashioned rooms. This is an important factor contributing to the increased protection against airborne infection in pre-1950 rooms, as will be discussed in section 2.4.4.

2.4.3 Mechanical ventilation delivered low rates of ventilation

In this study, an almost new mechanically ventilated facility delivered less than half the number of air-changes/hour recommended by guidelines.[112,113] On inspection of

the ventilation system, fan motors had not been maintained and fan blades were badly corroded and clogged with deposits. This was in part due to the absence of low grade particulate air filters to protect the air injection fans, which would in turn afford protection to the air extraction fans. An independent engineer assessed the ventilation system to be poorly designed, supplied with equipment of barely sufficient capacity to deliver the desired air-changes/hour, and lacking dampers to allow balancing of ventilation between rooms.

As a result the mechanical ventilation system was entirely re-furbished with completely new fans, new ductwork, the installation of air-flow dampers and new, improved placement of air injection and extraction vents. The placement of these extraction vents was aided by the use of computational fluid dynamics analysis of the ward.[144] However, despite these improvements, even with the recommended 12 air-changes/hour, absolute ventilation calculated for these mechanically ventilated rooms was considerably less than pre-1950 or modern naturally ventilated facilities: median 402 *vs.* 3769 *vs.* 1174 m^3/h respectively (median 12 *vs.* 40 *vs.* 17 ACH respectively).

Mechanical ventilation systems for the delivery of dilutional fresh air exchange and negative pressure are expensive to install, involve on-going running costs, and require a high standard of maintenance. Design, installation, and commissioning are specialist jobs and adequate maintenance also requires proper training and expertise. Filters must be changed regularly and a robust system must be in place for timely checks on the direction of airflow into negatively pressurised rooms. A saturated filter located before an air extractor fan quickly reduces the efficiency of that fan, allowing the build up of positive pressure in an isolation room if air supply fans continue to function normally. Indeed studies have demonstrated both inadequate rates of air

exchange and positive pressure to be a common finding in supposedly negative pressure isolation rooms.[122,130-132] As discussed in the introduction, numerous nosocomial outbreaks of TB in the developed world have been associated with failings in mechanical ventilation systems, through inadequate air exchange rates,[7,119] incorrect direction of airflow[6,8,12] or re-circulation of air.[6,12,16,128] These studies have all been conducted in the developed world, and good maintenance of such mechanical ventilation systems in low resource settings is likely to be less frequent.

2.4.4 Implications for the risk of airborne infection

It can be seen from the data presented that the theoretical risk of airborne tuberculosis infection was significantly less in older, spacious facilities with high-ceilings and plenty of fresh-air from large windows on more than one wall. In contrast, modern wards with low-ceilings and small-windows were associated with higher risk, and mechanically-ventilated rooms with sealed windows had the highest risk of all, even when ventilated optimally according to guidelines.

There are several limitations of the Wells-Riley equation as a model of airborne infection. The most obvious is that it assumes a constant, steady-state production of infectious quanta by the source case, which is implausible in the real world. The other major assumption is that infectious particles, once expelled by the source case, are instantly randomly distributed in the room air. Again this is implausible, with infectious droplet nuclei being found in greater concentration near the source, such that it is likely that a susceptible in close proximity to an infectious case is at greater risk of becoming infected than a susceptible on the other side of the room. In this way, a protective effect of dilution of infectious particles in larger room volumes may be

neglected by the Wells-Riley model. However, within these limitations, the Wells-Riley equation is an accepted model airborne infection risk, and in this study it is extremely useful as a tool to compare the risk of airborne contagion in pre-1950 and modern naturally ventilated facilities and mechanically ventilated facilities.

It is important to realise that the measure of ventilation included in models of airborne infection and which is a major determinant of the likelihood of infection is absolute ventilation (Q m^3/h in the Wells-Riley model). In contrast, most guidelines focus on air-changes/hour. Both measures of ventilation are valid and give information that the other does not, but focussing solely on air-changes/hour can be misleading. As discussed in chapter 1, fewer air-changes/hour are required in a large room compared with a small room to achieve a certain amount of absolute ventilation. The theoretical protection against airborne infection provided by ventilation would be identical in each room according to the Wells-Riley model, even though there would be fewer air-changes/hour in the larger room. If both rooms were ventilated at 12 ACH according to guidelines, the protection against airborne infection would be much greater in the room with the higher ceiling. There may in addition be a benefit from increased dilution of infectious particles in larger volume rooms (for example rooms with higher ceilings) not appreciated using the Wells-Riley equation as it assumes perfect air mixing and steady state conditions. Crowding of patients is of course important, and larger rooms are likely to house larger numbers of patients. However, increasing ceiling height increases room volume without affecting patient crowding (floor area per patient).

Thus the pre-1950 naturally ventilated rooms in this study gave protection against airborne infection not only through high rates of air-changes/hour, but also through

high rates of absolute ventilation. The pre-1950 rooms had similar patient crowding to modern post-1970 naturally ventilated rooms (9.2 *vs.* 9.3 m^2 floor area per patient; p=0.5) but owing to their higher ceilings had greater room volume per patient (median 39 *vs.* 27 m^3 per patient; p=0.004). With larger windows, greater air through-flow, and greater exposure to prevailing winds, these pre-1950 rooms had higher rates of ventilation combined with the additional advantage of larger volumes.

2.4.5 Advantages and disadvantages of natural *vs.* mechanical ventilation

Natural ventilation offers a number of advantages over mechanical ventilation. One of the most important advantages is financial: whilst mechanical ventilation is expensive to install and to maintain, natural ventilation offers high rates of air exchange for little or no cost. Heat loss from buildings can be an important factor as buildings are expensive to heat, but much tuberculosis occurs in the tropics where centrally heated buildings are uncommon.

The calculated risks of airborne tuberculosis transmission presented in this chapter demonstrated significantly reduced risk in naturally ventilated facilities, especially in those of old-fashioned design. The optimal 12 ACH of mechanical dilutional ventilation used in the model is in fact only recommended for high-risk settings such as negative-pressure isolation rooms and bronchoscopy suites. In the majority of the remaining areas in health care facilities, rates of mechanical ventilation (even when functioning properly) are much lower and based largely on odour control and comfort considerations.[145] In the model of airborne infection used in this chapter with the infectious source q=13 (the untreated office worker), 39% of susceptibles were predicted to become infected in mechanically ventilated respiratory isolation rooms at

12 ACH, compared with 33% in modern and 11% in pre-1950 naturally ventilated facilities. Were all these modern naturally ventilated hospital rooms in the study considered instead to be mechanically ventilated at 6 ACH (a relatively high rate for non high-risk areas in health care settings), the model predicted that 70% of susceptibles would become infected. Risks of transmission would increase further were mechanical ventilation systems to be poorly maintained.

Thus the second advantage of natural ventilation is its applicability to a wide range of healthcare settings. High air-exchange mechanical ventilation by its nature must be limited to certain high-risk areas, for example respiratory isolation rooms, infectious diseases wards, bronchoscopy suites, and intensive care units. However, the most infectious respiratory disease patients are likely to be undiagnosed, and therefore as yet untreated. These patients are most commonly found in out-patient waiting rooms, emergency departments, or undetected on general medical wards. This problem may be exacerbated by HIV, through increased susceptibility to TB, increased hospitalisation and health care seeking behaviours, diagnostic delay due to atypical presentation of disease, and poor response to treatment. There is evidence to suggest that unsuspected and undiagnosed TB cases represent a significant proportion of nosocomial transmission to health care workers in north America,[122,146] and in a Canadian study of 250 tuberculosis patients, 117 (47%) made a total of 258 pre-diagnosis visits to emergency departments, indicating the importance of such areas for TB transmission.[147] This study has shown that natural ventilation can provide high rates of ventilation in a variety of settings: 70 different rooms were studied including emergency departments, out-patient consulting rooms, respiratory isolation rooms, and general medical wards. In addition, natural ventilation is applicable in other health

care settings not included in this study, such as primary health care waiting rooms, and health posts in low resource settings. Furthermore, natural ventilation is applicable in non-healthcare settings where infectious individuals and susceptibles are clustered such as prisons and homeless shelters.

A well designed mechanical ventilation system delivering adequate air exchanges and negative pressure is undoubtedly of great use in efforts to control the nosocomial transmission of airborne disease. However, there is a limit to the number of air exchanges that such systems can deliver, for reasons of cost, vibration of fans and motors, noise, and draughts caused by high air-flows emanating from vents. In rooms with large windows, very high rates of air exchange are easily attained, even on relatively windless days. This is achieved by almost piston-like flow of air through large windows, across a room to open windows on opposite walls, and large volumes of air can be moved without uncomfortable draughts.

There is a natural inclination to wish to 'contain' infectious air – 'to shut the germs in'. However, the enormous dilution resulting from release of contaminated air into the atmosphere prevents natural ventilation from 'contaminating' the immediate environment significantly. In addition, there is a powerful sterilising effect of natural UV light. Whilst exhaust air from TB isolation rooms may be filtered, air from general clinical spaces is usually pumped unfiltered into the atmosphere. Consequently, opening the windows releases the same number of infectious particles into the atmosphere as mechanical ventilation without causing any significant risk to those outside, but does so with greater protection for people inside the rooms. A disadvantage of natural ventilation is the difficulty in controlling direction of airflow due to the absence of negative pressure. Contamination of corridors and adjacent

rooms is therefore a risk, particularly on completely still days. However, it is possible to locate a tuberculosis ward, for example, on the uppermost floor of a building and downwind of other rooms or the nursing station. Furthermore, corridors that are open at both ends may allow the passage of large volumes of fresh air that may compensate for the absence of negative pressure. Whilst it has already been noted that poorly maintained negative pressure facilities may in fact deliver positive pressure,[122,130] it should also be noted that even when negative pressure rooms function properly, air volume migration out of the room occurs during entry or exit through the door.[148]

Further potential disadvantages of natural ventilation include noise pollution, for example from road traffic. Another is security, but metal bars can provide a secure barrier without impeding the flow of air. This is of particular importance in prisons, where overcrowding and tuberculosis transmission are major problems. Even in warm climates, it may be cold at night, especially at certain times of the year, and large windows may get closed by occupants. However, this study has demonstrated that significant levels of ventilation can be achieved with only a proportion of windows open. Indeed visitors and staff, the main groups at risk of nosocomial infection, are less likely to be sharing ward spaces with patients at night. In addition, supplementary measures for the control of TB transmission such as upper room germicidal ultraviolet light may be employed during night time hours.[112]

Airborne infections may be prevented by screening individuals for infectiousness and isolating contagious patients in individual negative-pressure rooms in which carers and visitors always wear high quality particulate respirators. However, respirator efficacy depends on a good fit which is not always easily achieved.[117] In addition, use of respirators is stigmatising to patients, and it is often difficult to ensure strict

compliance with respirator policies by all the visitors to a ward, including health care workers.[42,118] Respirators are often not used when patient infectiousness is undiagnosed, such as in waiting rooms and emergency departments, and as discussed it is often these untreated patients who are the most infectious. Furthermore, their expense precludes widespread use in many resource-limited settings where burdens of airborne disease such as tuberculosis are high.

2.4.6 Conclusion

This research demonstrates that protective ventilation may be maximised by opening windows and doors and emulating hospital design from 50 years ago. Natural ventilation offers advantages over mechanical ventilation in the prevention of the institutional transmission of tuberculosis, especially in low resource settings. When designing medical facilities there are lessons to be learnt from the past and it may be better to replace over-crowding and poor ventilation with the safer design principles of our predecessors. When infectious and susceptible individuals must share rooms and respirators and negative-pressure isolation are impractical, crowding should be reduced and windows and doors opened to maximise natural ventilation and reduce the risk of airborne contagion.

Natural ventilation is not suited to cold climates however, and even in warm climates there is a tendency to close windows at night. There is therefore a need for the evaluation and validation of alternative environmental controls for the prevention of airborne TB transmission, particularly for use in low resource settings. Evaluation of such interventions requires a means to measure TB transmission, and this is the subject of the following chapter.

	Air-changes/hour (log_{10})		Absolute ventilation (m^3/h) (log_{10})		Calculated risk of TB transmission (log_{10})	
	Coefficient	p	Coefficient	p	Coefficient	*p*
Area windows a/o doors open (m^2)	0.029	<0.001	0.036	<0.001	-0.029	<0.001
Wind speed (km/h)	0.047	<0.001	0.049	<0.001	-0.036	<0.001
Air through-flow	0.217	<0.001	0.188	0.039	-0.112	0.06
Ceiling height (m)	0.086	0.007	0.149	<0.001	-0.163	<0.001
Floor area (m^2)	-0.006	<0.001	0.005	<0.001	0.006	<0.001
Season	0.029	0.36	0.042	0.34	0.028	0.43
Height of room above ground (m)	0.004	0.25	0.006	0.18	0.002	0.62

Table 2-1: Architectural and environmental determinants of natural ventilation and protection against airborne infection

Ventilation was measured as ACH and as absolute ventilation (m^3/h), and protection against airborne infection was estimated by the calculated risk of airborne TB transmission for 24 hour exposure to TB cases producing 13 infectious quanta/hour.[125] Regression analyses were performed using all data for all naturally ventilated rooms in experiments where at least one window or door was opened.

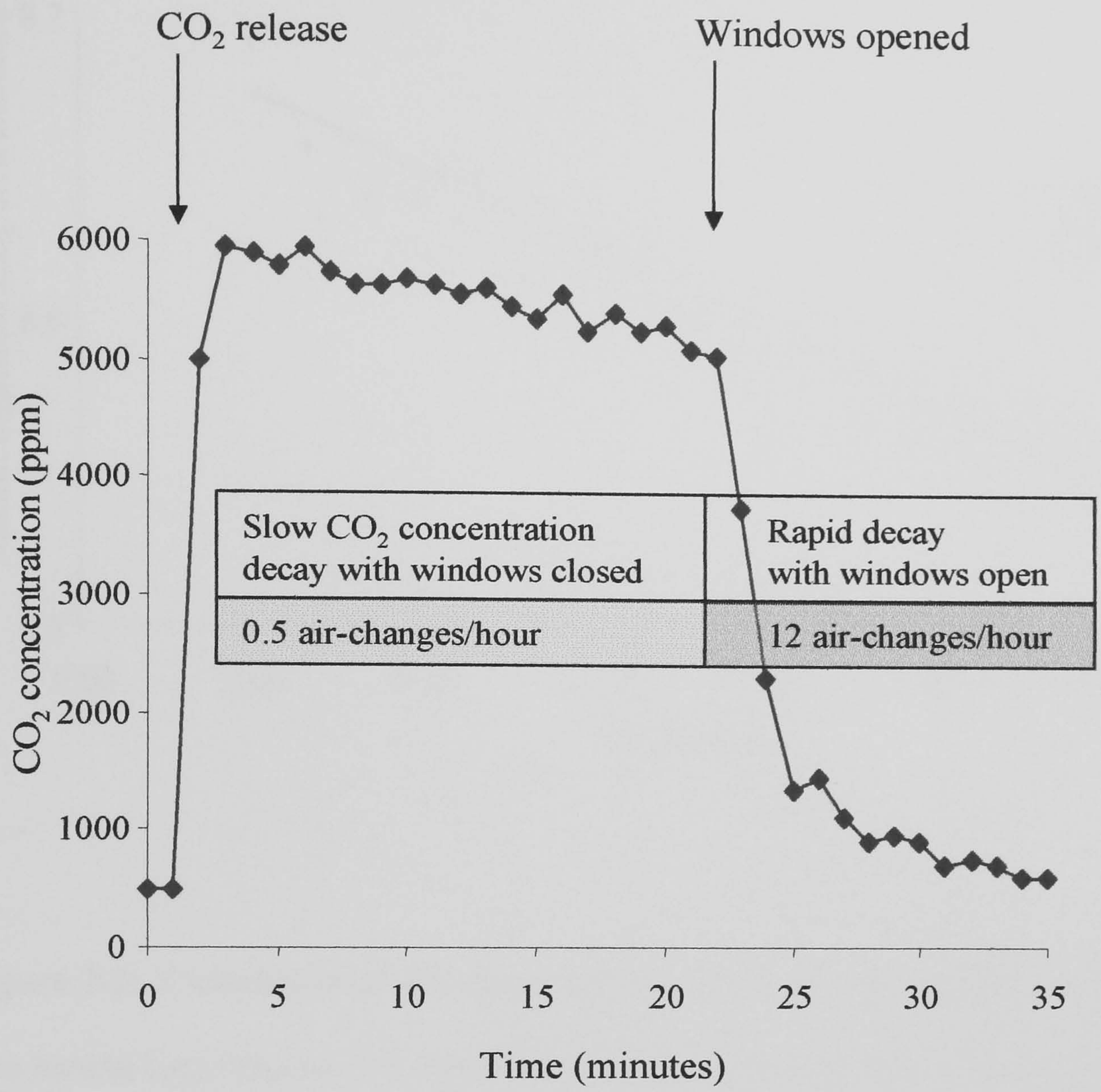


Figure 2-1: A typical carbon dioxide concentration-decay graph

Baseline CO_2 levels were 500 ppm, rising to 6000 ppm following gas release. For the next 20 minutes CO_2 concentration decayed slowly whilst all windows and doors remained closed. A rapid reduction in CO_2 concentration was seen immediately following the opening of windows at time point 22 minutes. This corresponded to the increase in natural ventilation as a result of opening the windows.

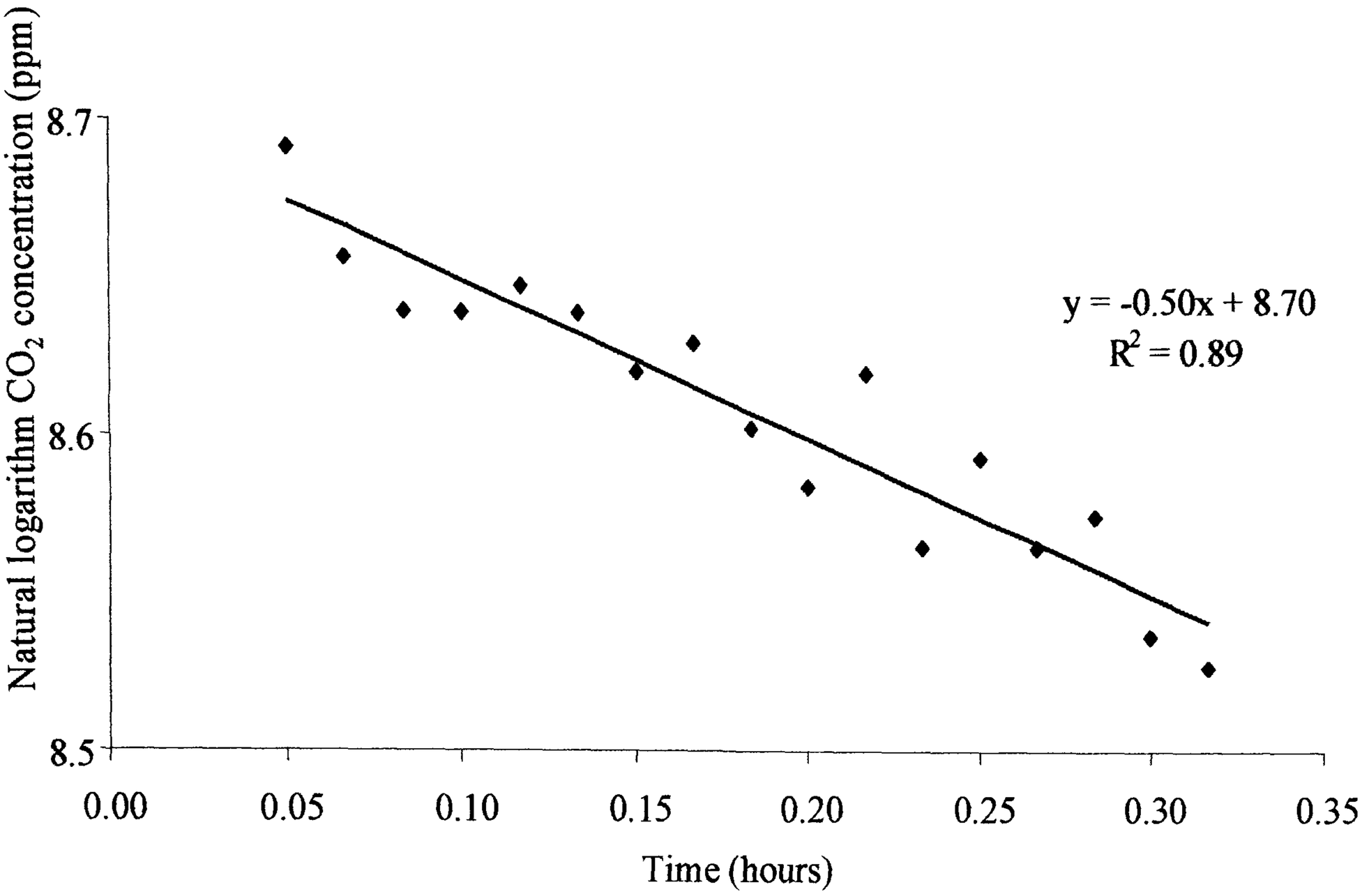


Figure 2-2: Calculation of air-changes/hour with windows and doors closed

The natural logarithm of CO_2 concentration (ppm) was plotted against time (hours) for the period with windows and doors closed after air mixing with fans. The line of best fit was drawn through the points. Air-changes/hour were calculated as the positive value for the gradient of the line, in this case 0.5 ACH.

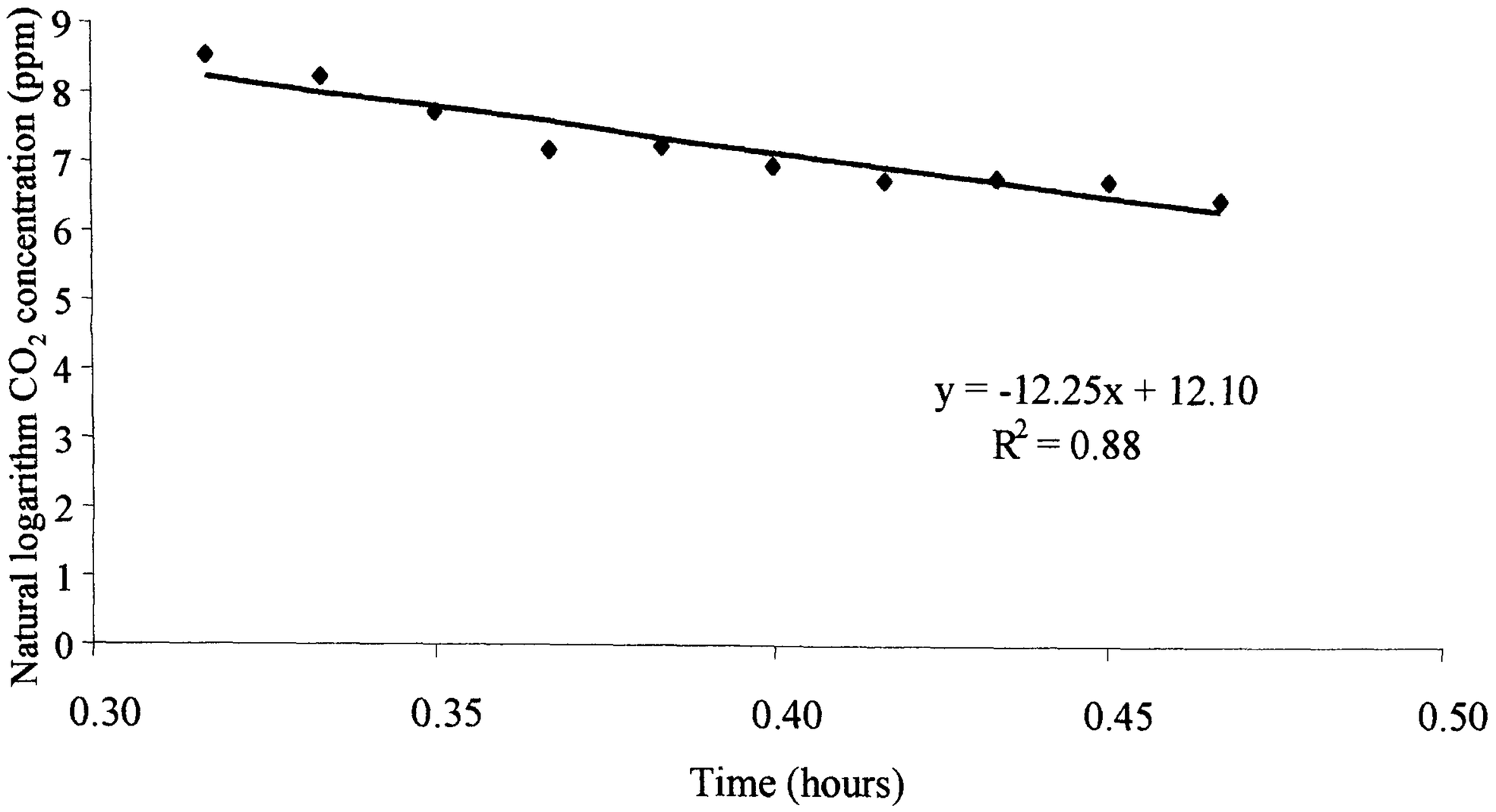


Figure 2-3: Calculation of air-changes/hour with windows and doors open

The natural logarithm of CO_2 concentration (ppm) was plotted against time (hours) for the period immediately following the simultaneous opening of windows and doors until concentration reached 200 ppm of baseline. The line of best fit was drawn through the points. Air-changes/hour were calculated as the positive value for the gradient of the line, in this case 12 ACH.

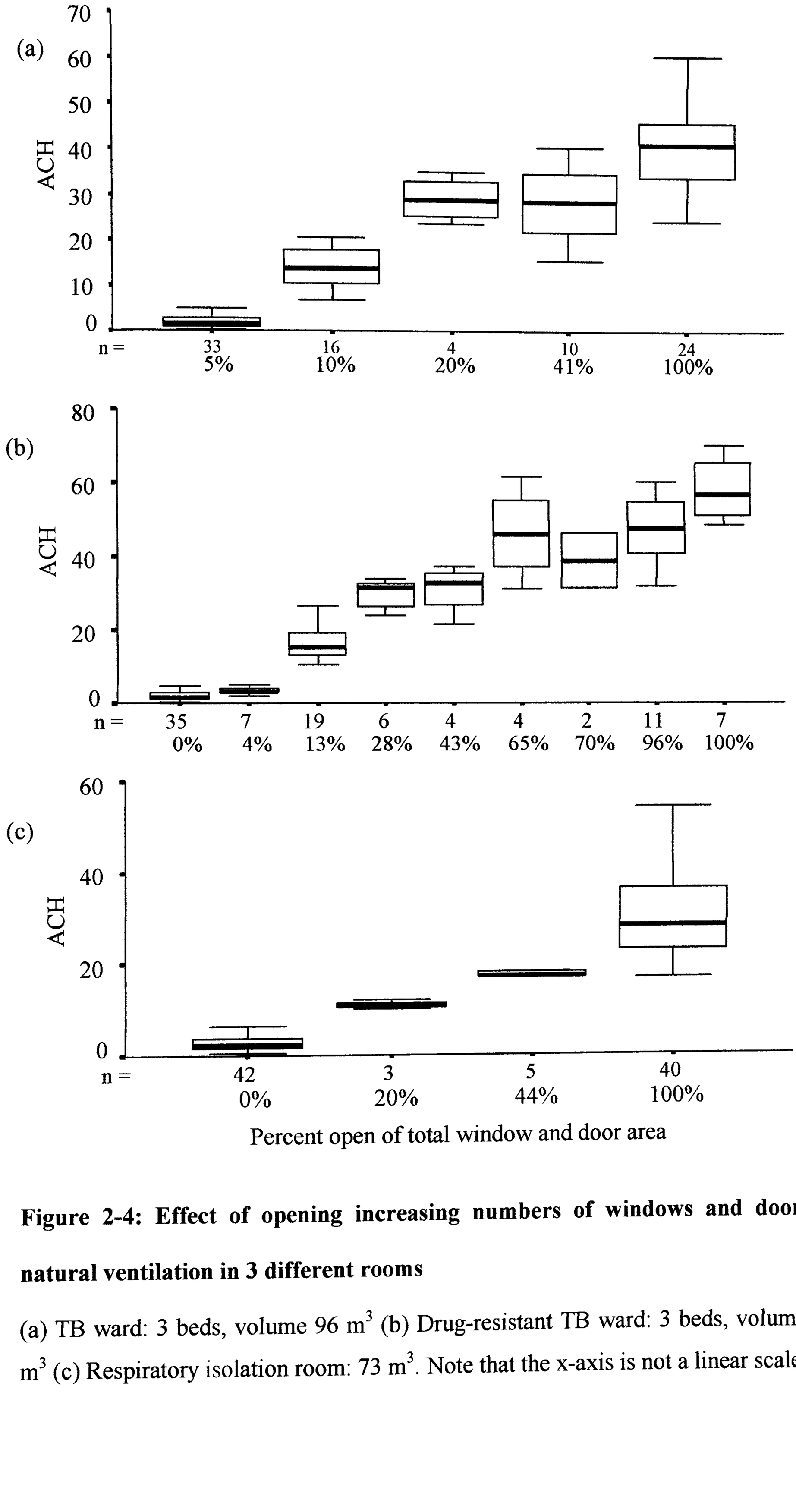


Figure 2-4: Effect of opening increasing numbers of windows and doors on natural ventilation in 3 different rooms

(a) TB ward: 3 beds, volume 96 m^3 (b) Drug-resistant TB ward: 3 beds, volume 147 m^3 (c) Respiratory isolation room: 73 m^3. Note that the x-axis is not a linear scale.

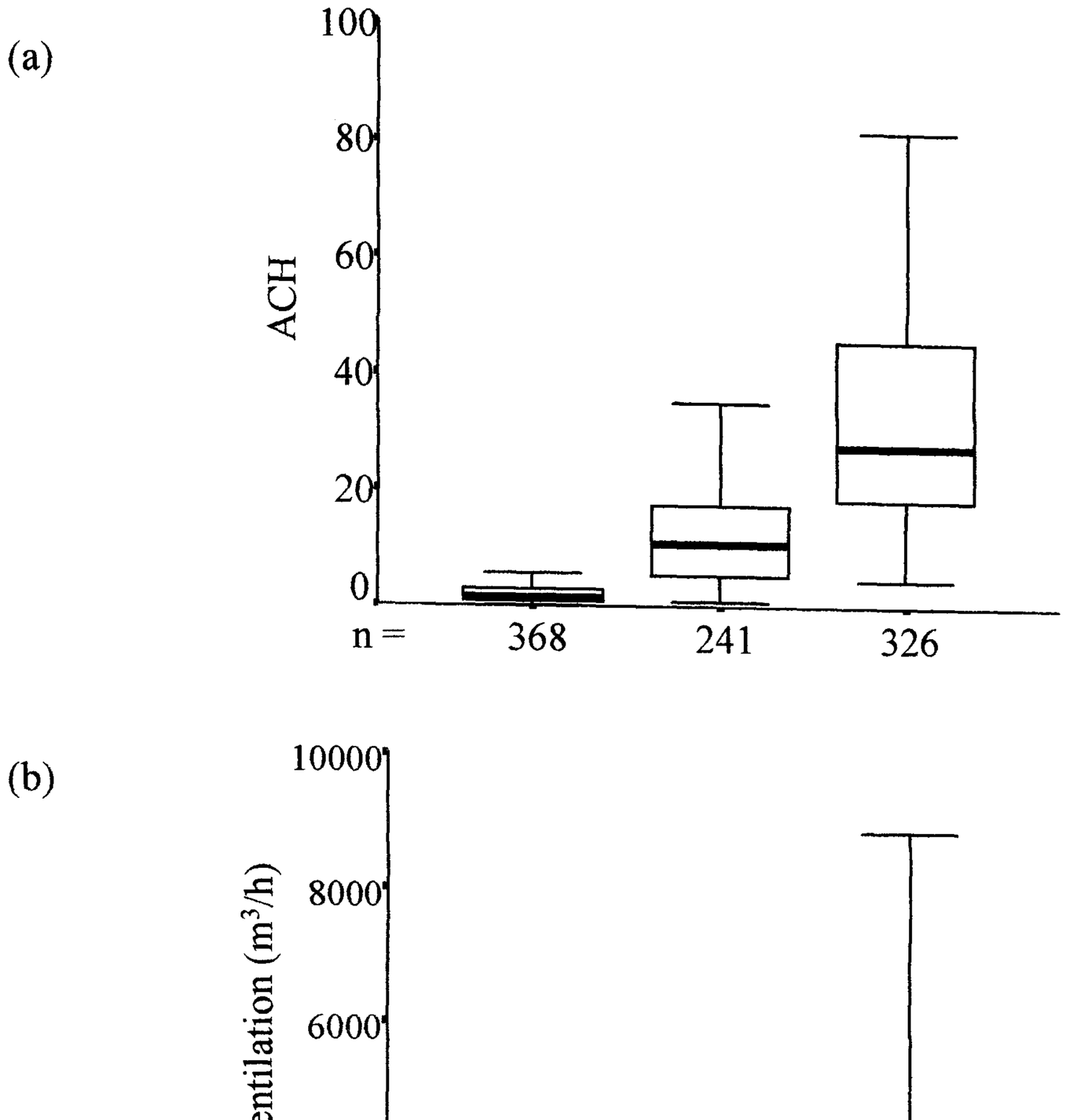


Figure 2-5: Effect of opening increasing numbers of windows and doors on natural ventilation measured as (a) ACH (b) Absolute ventilation (m^3/h)

All data from 368 experiments in 70 different naturally ventilated rooms are included. 'n' denotes the number of experiments measuring ventilation. 'Closed' denotes all windows and doors closed, 'Partially open' denotes some but not all of windows and/or doors open, and 'Fully open' denotes all windows and doors open. In some rooms it was not possible to open all of the windows and doors and this accounts for 'n' being smaller for 'Fully open' compared with 'Closed' experiments.

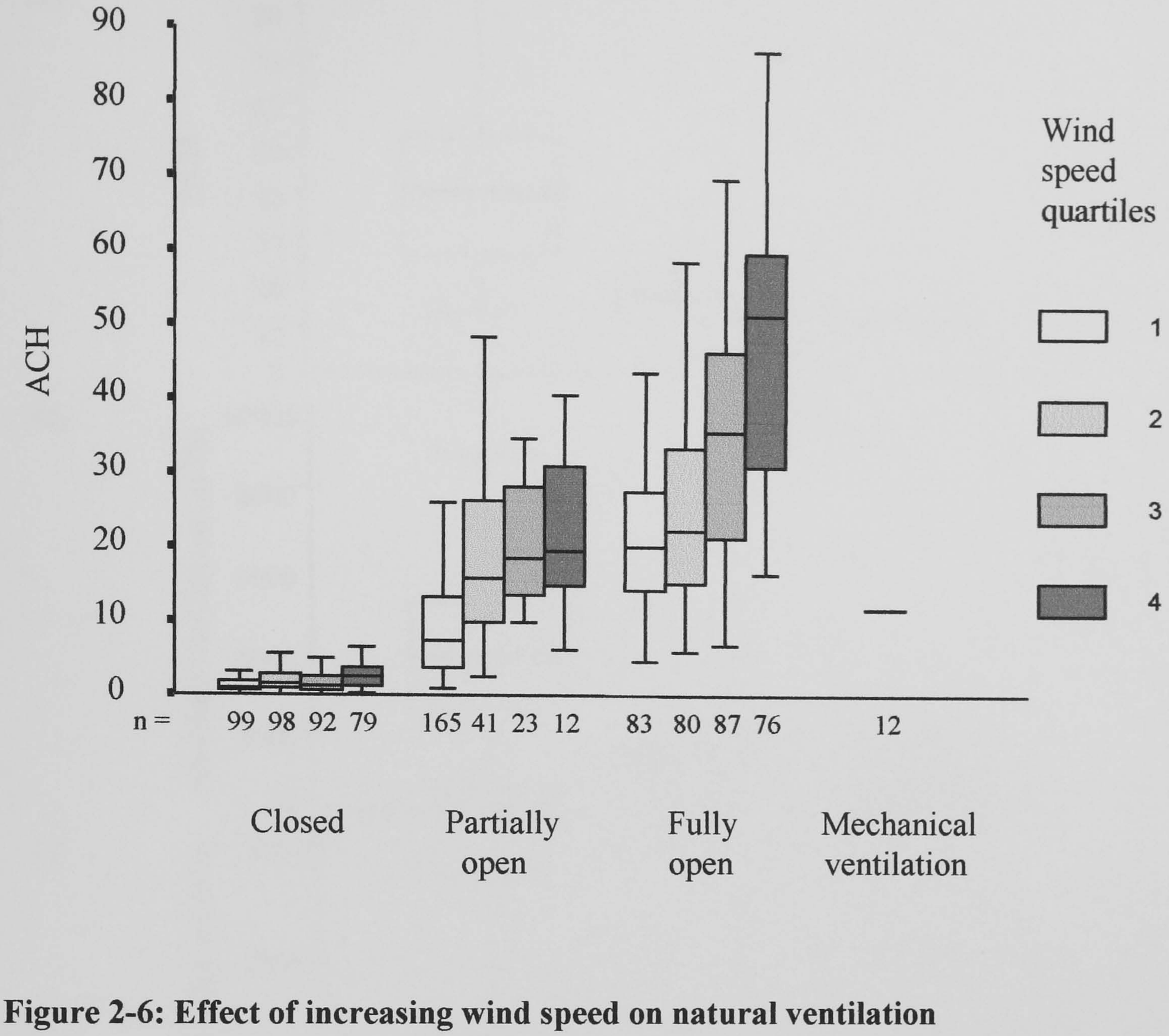


Figure 2-6: Effect of increasing wind speed on natural ventilation

Natural ventilation measured in ACH is shown for 'Closed', 'Partially open' and 'Fully open' naturally ventilated facilities, compared with mechanically ventilated wards ventilated at the recommended 12 ACH. Measurements for 'Closed', 'Partially open' and 'Fully open' are subdivided into four colour coded bars representing wind speed quartiles. 'n' denotes the number of ventilation experiments measuring air-changes/hour, or the number of rooms in the case of mechanical ventilation. The lowest quartile of wind speeds ('1') were <2.0 km/h, the second quartile ('2') were 2.0-2.8 km/h, the third quartile ('3') were 2.9-5.5 km/h and the highest quartile ('4') were >5.5 km/h.

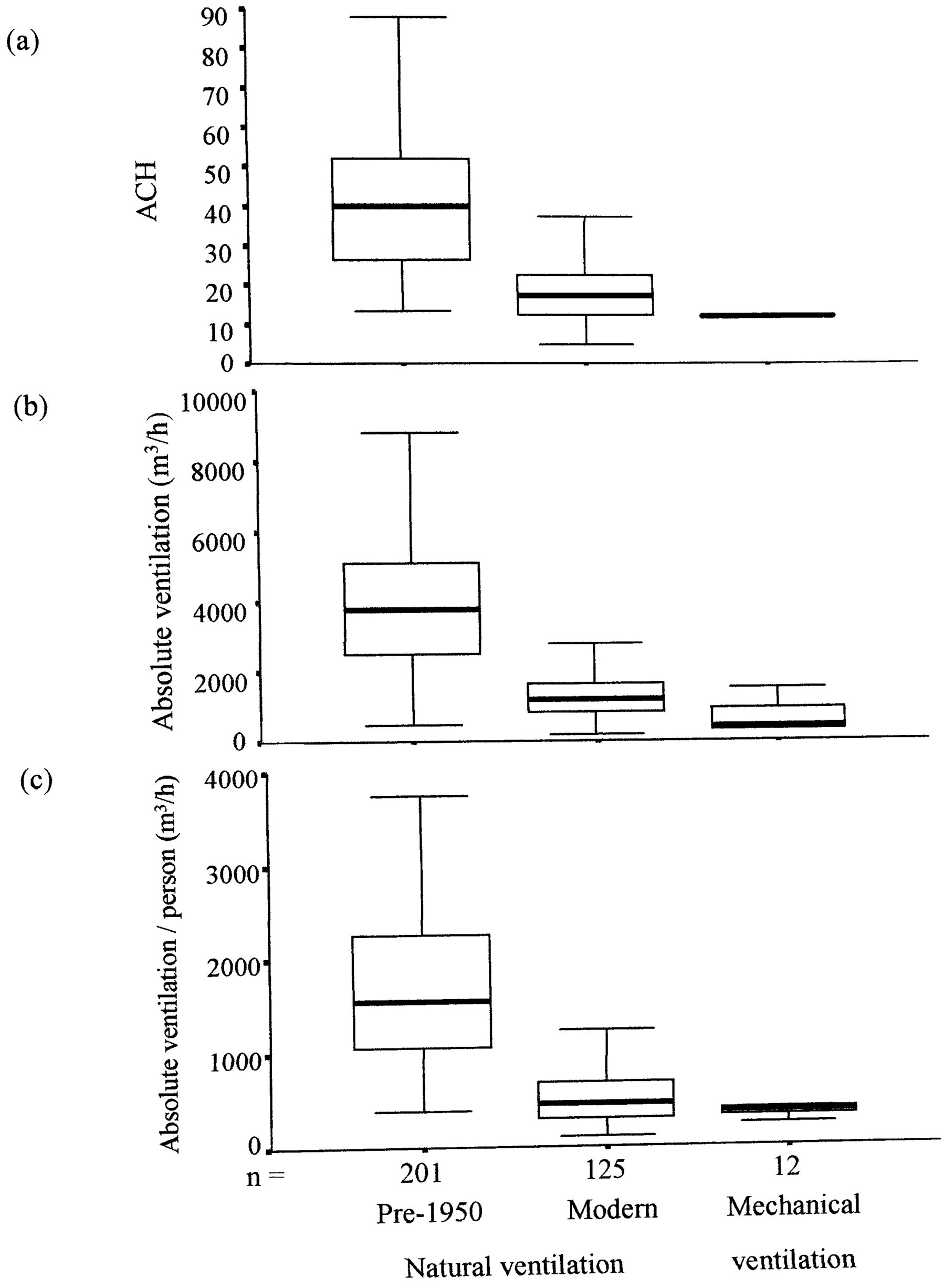


Figure 2-7: Comparison of pre-1950 and modern naturally ventilated facilities with windows and doors fully open, with mechanically ventilated respiratory isolation facilities ventilated at the recommended 12 ACH.

Ventilation is expressed as (a) ACH (b) Absolute ventilation (m^3/h) and (c) Absolute ventilation per person (m^3/h) for each category of room. 'n' denotes the number of measurements, or calculations for the mechanically ventilated rooms at 12 ACH.

(a)

(b)

Figure 2-8: Poor maintenance of a mechanical ventilation system

A poorly maintained (a) air extraction fan and (b) air supply fan with corroded fan blades clogged with deposits.

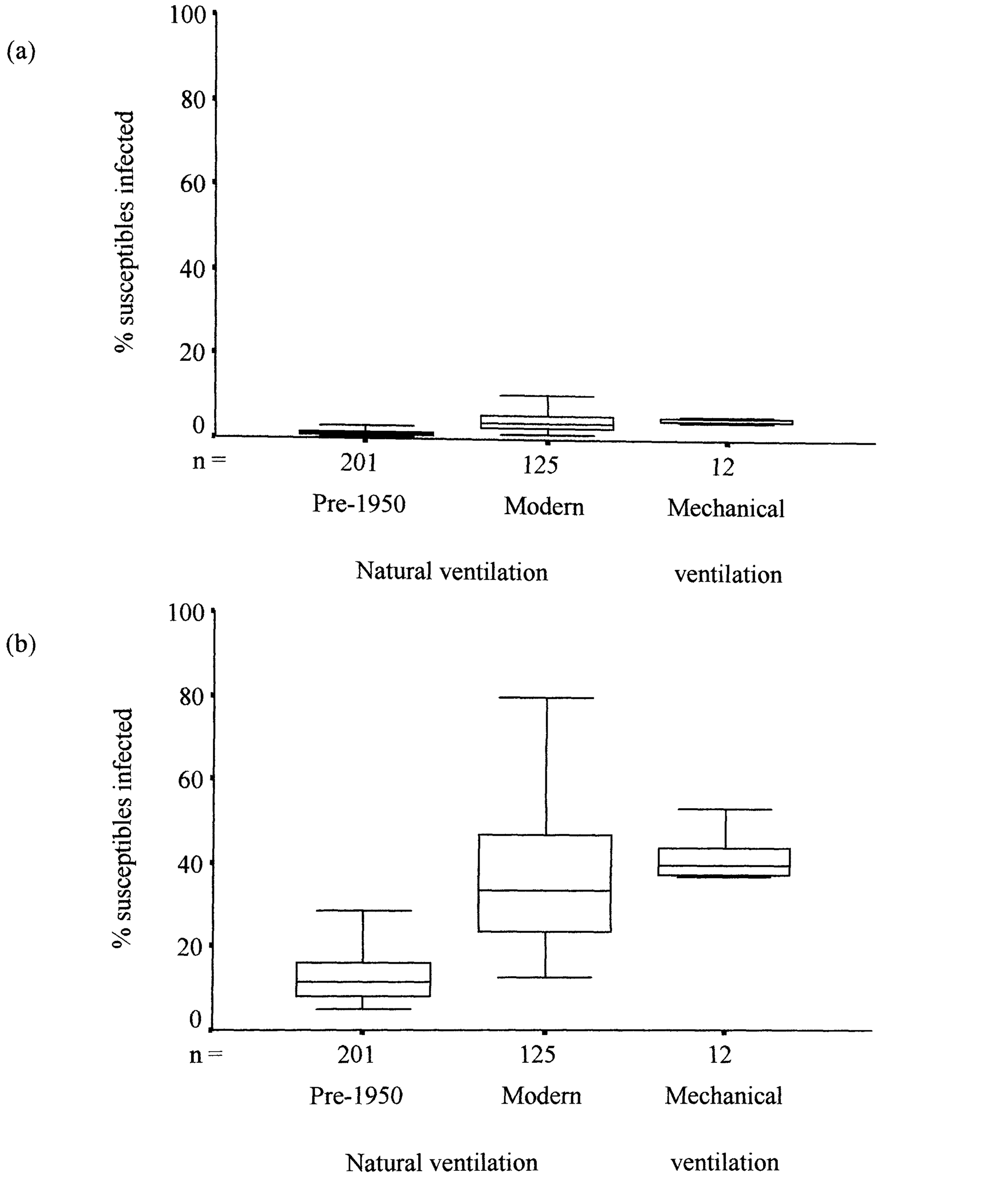
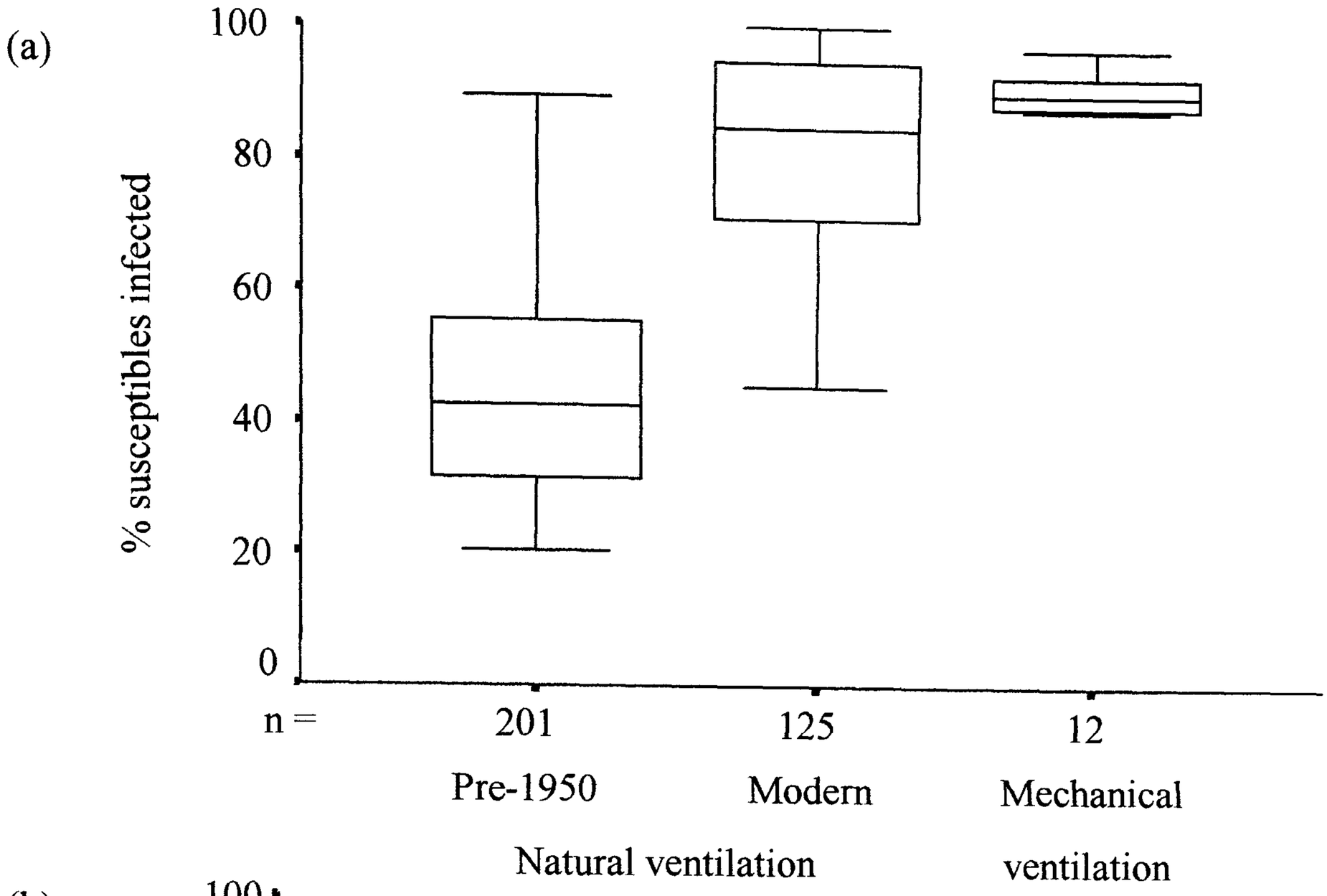


Figure 2-9: Modelling of airborne TB transmission risk in naturally ventilated facilities *vs.* mechanically ventilated respiratory isolation rooms at 12 ACH.

The percent of susceptibles infected is shown for: (a) 24 hour exposure to TB cases producing 1.3 infectious quanta/hour[123] (b) 24 hour exposure to TB cases producing 13 infectious quanta/hour.[125] 'n' denotes the number of calculations.



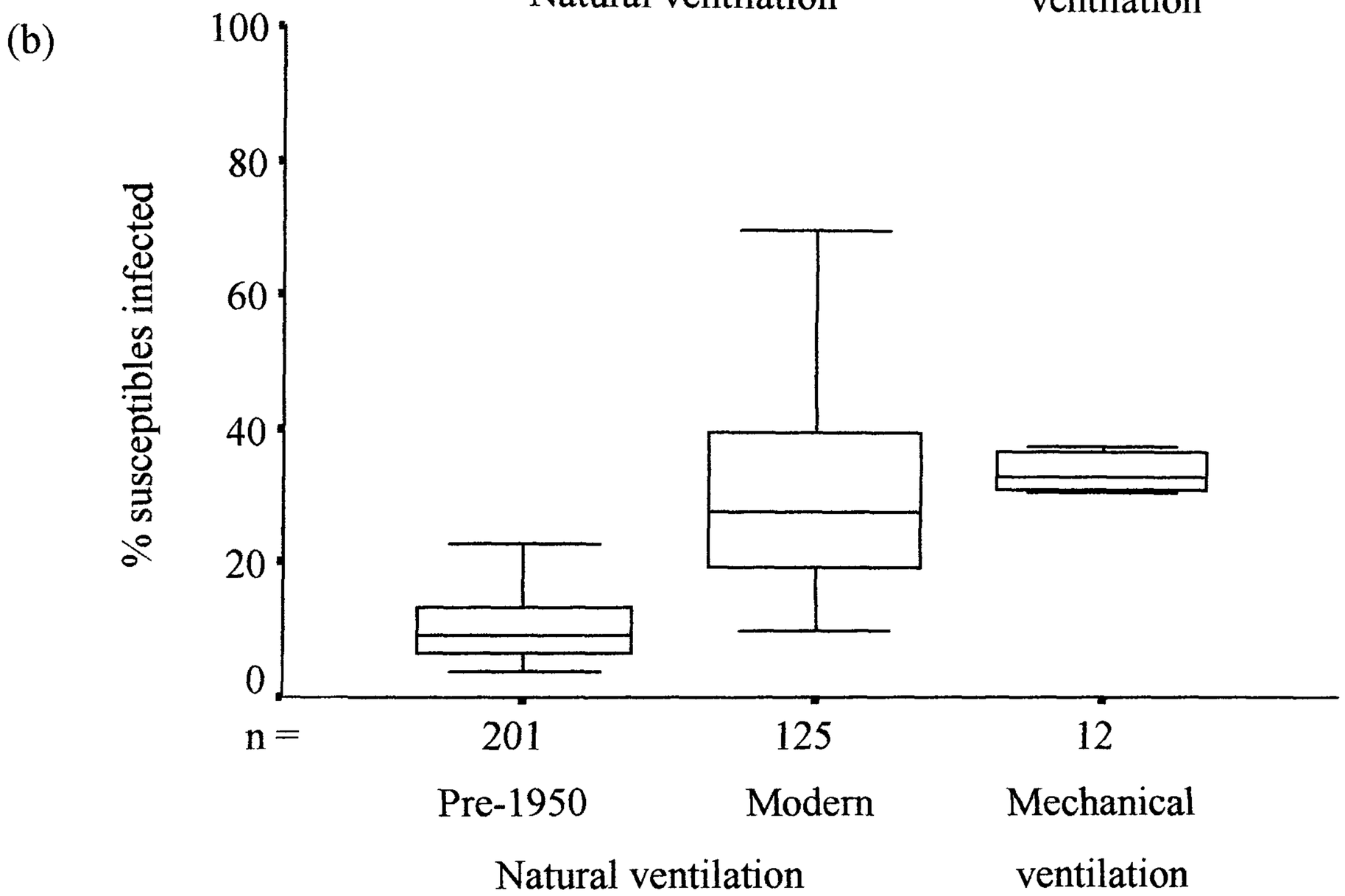


Figure 2-10: Modelling of airborne TB transmission risk in naturally ventilated facilities *vs.* mechanically ventilated respiratory isolation rooms at 12 ACH.

The percent of susceptibles infected is shown for: (a) 24 hour exposure to cases of laryngeal TB producing 60 infectious quanta/hour.[123] (b) One hour exposure to bronchoscopy cases generating 250 infectious quanta/hour.[7,123] 'n' denotes the number of calculations.

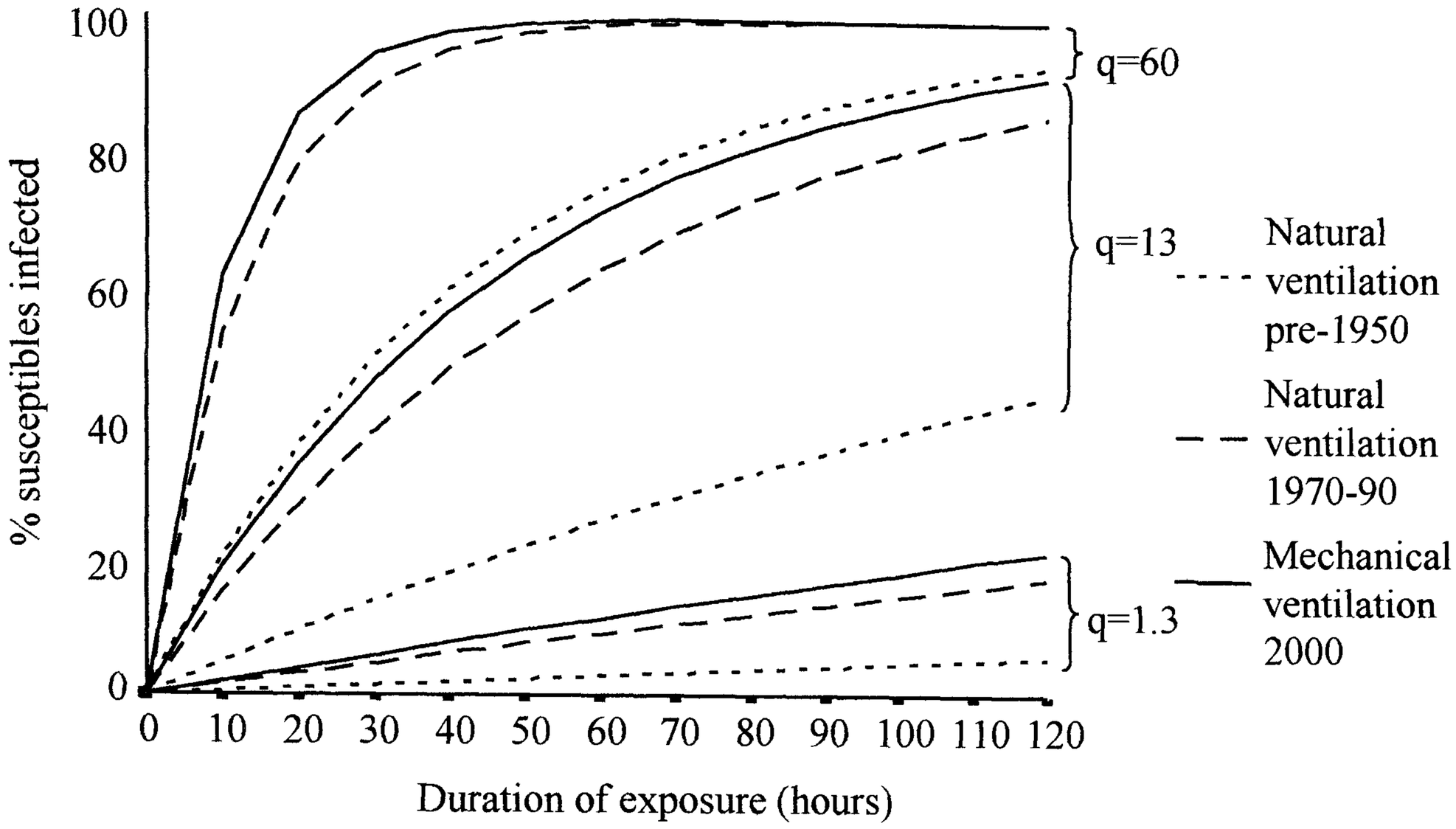


Figure 2-11: Modelling of airborne TB transmission risk over time

Theoretical risk of TB infection over time for exposure to TB source cases of different infectiousness in pre-1950 naturally ventilated facilities *vs.* modern post-1970 naturally ventilated facilities *vs.* mechanically ventilated facilities at 12 ACH. The infectious sources are: q=1.3, standard ward TB patients studied by Riley;[123] q=13, an untreated TB case who infected 27 co-workers in an office over 4 weeks;[125] q=60, the most infectious case studied by Riley, a case of laryngeal TB.[123] Median values for all measures of absolute ventilation for each category of naturally ventilated room were used in the model. Respiratory isolation rooms were assumed to be mechanically ventilated at the recommended level of 12 ACH (see text). It can be seen that the protective effect of ventilation against airborne infection decreases as the infectiousness of the source increases. It can also be seen that according to the model all susceptibles would eventually become infected with sufficiently long duration of exposure.

CHAPTER 3

A guinea pig model to detect airborne tuberculosis transmission

3.1 Introduction

The classic studies of Riley and colleagues from the 1950s-60s have already been briefly described.[73-76] An average 125 guinea pigs resided over four years in a chamber above six TB isolation rooms at the Baltimore Veterans Administration Hospital, USA. Exhaust air from the ward passed over the guinea pigs, resulting in 134 guinea pig infections, of which the human source was demonstrated in most cases by using temporal exposure patterns and the drug susceptibility profiles of patient and animal *M. tuberculosis* strains. These studies demonstrated airborne TB transmission by particles that could remain suspended in the air and travel from the ward through ducts to the guinea pig exposure chamber, characteristics of the droplet nuclei postulated by Wells to be responsible for airborne tuberculosis transmission.[69] These experiments demonstrated a great variability in infectiousness of individual patients, a reduction in infectiousness after the initiation of effective antimicrobial chemotherapy and air disinfection by placement of UV lights in ducts.[73-76]

In order to evaluate strategies for the control of institutional TB transmission, such transmission must in some way be measured. Transmission to HCWs is one possible outcome measure, and new diagnostic tests for latent TB infection that have greater

specificity than the tuberculin skin test make such studies more feasible than previously.[149-152] However, these studies remain logistically difficult owing to staff turnover, and are susceptible to important confounding factors such as HCW exposures to TB in other parts of a hospital where an intervention is not in place, or TB exposures outside the workplace.

Directly sampling the air for *M. tuberculosis* would be the most obvious method to evaluate the effectiveness of an intervention to prevent TB transmission. Unfortunately, there have been no successful attempts at the quantitative recovery of live mycobacteria from air.[153,154] Conventional mechanical air sampling is made difficult for TB by the paucity of airborne organisms,[76] damage through the physical stresses of sampling, and fungal overgrowth of samples.[155] PCR has recently been used to detect TB DNA from air filters,[138,156] but this assay is not quantitative and detects non-viable as well as infectious particles. Thus the guinea pig remains the gold standard for the detection of airborne *M. tuberculosis*, and hence this animal air sampling model was chosen for this project despite the logistical difficulties involved. A re-creation of Riley's classic studies using modern molecular tools in today's era of HIV infection and multidrug-resistant TB forms the core of this thesis. The first step was to design and build a guinea pig air sampling facility on the roof of a TB ward, and demonstrate that this experimental facility could be used to detect airborne tuberculosis transmission.

3.2 Methods

3.2.1 Setting

The Servicio de Enfermedades Infecciosas y Tropicales at Hospital Nacional Dos de Mayo in Lima, Peru was selected for this work. An old fashioned TB ward with high ceilings in this department had been converted in the year 2000 into Peru's first facility for TB patients providing mechanical ventilation and negative pressure. The ventilation system was designed to deliver dilutional ventilation of 6 ACH, to two four-bedded rooms, a six-bedded room and an isolation room. This facility promised a high throughput of tuberculosis patients, with a significant proportion of drug-resistant TB. Indeed it was the high rates of nosocomial transmission of drug-resistant TB in this HIV-TB service that originally prompted the construction of this negative-pressure facility.[157] The roof of the building was large and empty, another important factor in the selection of this site for the study. All air extracted from the ward originally passed unfiltered into the atmosphere through three meter high chimneys on this roof.

3.2.2 Animal air sampling facility design

An airtight animal facility 9.8 m long x 2.3 m wide x 1.8 m tall was constructed on the roof of the TB ward as shown in Figures 3.1 & 3.2. Air from each of the two four-bedded rooms arrived on the roof via one of the two ducts shown, passed through the guinea pig house, over one of the two extractor fans seen in the picture, and out of the exhaust chimney. This avoided the possibility of disruption or precipitation from the air of droplet nuclei by the high-speed extractor fan blades, which might have reduced

the infectivity of the air before it passed over the guinea pigs had the fans been sited 'upstream' of the animals. The air from the six-bedded room (which was largely empty) and the single isolation room was exhausted directly into the atmosphere before passing over the guinea pigs, through a chimney the top of which may just be seen in the right hand side of the photograph in Figure 3.1. The elevation of the roof of this building is 10 m from the ground, and the roof is well exposed to Lima's prevailing winds which come from the Pacific Ocean. The building is not close to other buildings, indeed it was built in the 1940s specifically as an isolated block for tuberculosis patients. Considering the enormous dilution of infectious particles on leaving the chimneys, it was felt unnecessary to alter the hospital's original policy to exhaust air unfiltered into the atmosphere.

The animal house was constructed with a division down the middle, to separate the colony into two halves. A schematic diagram of the facility is shown in Figure 4.8 in Chapter 4. The aim of this division was to minimise the risk of a potential outbreak of bacterial diseases such as salmonellosis affecting the entire colony. Similarly, this reduced the possibility that horizontal spread of TB between guinea pigs could affect the whole colony. Air entered the facility through ducts centred on the midline, such that each half of the animal house received approximately equal proportions of air from the ward. Cages were constructed to house animals in groups of 6-8, with wire mesh bottoms to allow drop-through of faeces and urine to reduce the possibility of horizontal transmission of tuberculosis via the faecal-oral route.[158]

3.2.3 Animal air sampling facility commissioning

Ventilation was measured in the ward using a balometer capture hood (Alnor Lo-Flo Balometer, TSI Incorporated, Shoreview, USA) at air injection and extraction vents. Negative pressure on the ward was continuously measured using manometers (Model 25 Mark 2, Dwyer Instruments Inc, Michigan City, USA) and confirmed using smoke tubes (Allegro industries, Garden Grove, USA) to assess air flow direction around and under doors. Air flow in the animal house was measured using the same techniques. Air flow patterns within the animal house were assessed visually with smoke patterns resulting from the placement of smoke emitters in the air extract ducts of the ward (Regin HVAC Products, Shelton, USA). Leakage around doors, windows and through ductwork was also evaluated with smoke tests. The results of commissioning are reported in the next chapter as they are most relevant to calculations of patient infectiousness.

3.2.4 Animal source

8 week old male and female out-bred Peruvian guinea pigs were purchased from the breeding station of the Faculty of Veterinary Medicine of Universidad Nacional Mayor San Marcos. This was situated at 3200 m above sea level in the Mantaro valley in the central Andes of Peru. Animals were fed alfalfa and wild grass in accordance with veterinary school practice. Animals were identified by description and laser etched ear tags.

3.2.5 Animal quarantine

Animals were maintained in a specially constructed quarantine facility with restricted access at the breeding station in the mountains. The animal handler did not have TB or a history of TB, or any known contact with TB cases. Animals were kept in quarantine for at least one month before transfer to the animal facility on the hospital roof in Lima. This allowed at least two monthly purified protein derivative (PPD) skin tests to be performed (see next section) to ensure that the guinea pigs were completely free from tuberculosis infection before transfer to the air sampling facility.

3.2.6 Monthly PPD skin testing

Guinea pigs were tested at monthly intervals with 100 Units of PPD (PPD Tuberculin, Evans Vaccines Ltd, Liverpool, UK). 1 ml vials of 100,000 Units PPD were diluted to 1000 Units in 1 ml using the constituents in the manufacturer's instructions: glycerol 20.00% v/v; phenol 0.25% w/v; Tween 80 0.005% v/v; KH_2PO_4 0.138% w/v; Na_2HPO_4 0.724% w/v; NaCl 0.457% w/v; water for injection 100% v/v. 0.1 ml of diluted PPD was injected intra-dermally using 1 ml syringes with 26G needles. This was equivalent to the 0.002 mg of old tuberculin as used by Riley.[159] A small patch of hair on the flank of a guinea pig was first shaved, and depilatory cream (Veet, Reckitt Benckiser, Hull, UK) applied for a period of 10 minutes before being washed off to leave a patch of smooth, hairless skin exposed. All PPD skin test reactions in animals exposed to ward air were read by the PhD author. Reaction size (diameter of induration in mm) was initially recorded at 48 hours, in accordance with Riley's work.[159] Subsequently a comparison was made of reaction size at 24 compared with 48 hours. Following analysis of these results, all skin tests were read at both 24 and 48

hours. Positive PPD reactors were removed from the animal house and isolated before humane sacrifice and autopsy. A specially designed secure isolation enclosure with high levels of natural ventilation and individual cages was constructed at the opposite end of the roof to house these PPD positive animals before autopsy.

3.2.7 Animal maintenance and hygiene in the animal house

Animals were fed dry pellet feed, and water ad libitum. Dry pellet feed in place of alfalfa was used to reduce the risk of epidemic non-TB infection amongst the guinea pigs. Strict standards of animal hygiene were maintained, and daily care was shared by a qualified veterinary surgeon and the research fellow. Staff wore high quality half-face piece particulate respirators and protective clothing at all times in the animal house. Particulate respirator filters were changed at two monthly intervals. Waste was disinfected and removed for safe disposal on a weekly basis.

3.2.8 Unexposed PPD-negative control guinea pigs

An additional animal enclosure was built to house an average 40 negative control guinea pigs that breathed only fresh, uncontaminated outside air. This facility was naturally ventilated and situated at least 15 m up-wind of the exhaust chimneys of the main animal house. Care of negative control unexposed animals was identical to that of the exposed group. They are referred to as 'Unexposed PPD negative controls.'

3.2.9 Unexposed PPD-positive control guinea pigs

A group of 13 healthy, uninfected control animals were maintained in the same separate enclosure as the unexposed PPD negative controls. These animals were

injected by the intra-muscular route with 0.5 ml *Mycobacterium bovis* sensitising agent (Center for Veterinary Biologics Laboratory, Iowa, USA) as used in biological assays for the standardisation of batches of PPD.[160] PPD skin tests were performed monthly in the same way as the other groups of animals. This group performed the role of PPD positive controls, allowing verification of the efficacy of different batches of PPD used and to control for errors in the PPD dilution process. They are referred to as "*M. bovis* Positive Controls." Care of these animals was the same as that of the exposed group. A repeat injection of 0.5 ml *Mycobacterium bovis* sensitising agent was given if animals developed small PPD skin test responses (<7.5 mm).

3.2.10 Autopsy procedures

PPD positive animals and negative controls were sacrificed by intra-peritoneal injection with 2 ml pentobarbital sodium (65 mg/ml; Halatal; Montana S.A., Lima, Peru) confirmed by cervical fracture. Pentobarbital sodium has been used in many studies of TB in guinea pigs, and is not thought to affect mycobacterial growth.[161] These animals and any intercurrent deaths were double bagged and transported in sealed containers to the mycobacteriology laboratory at Universidad Peruana Cayetano Heredia in Lima.

All autopsies were performed using aseptic technique in a class II safety cabinet. Guinea pig hair over the abdomen and thorax was shaved, and remaining hair removed with depilatory cream. Skin was sterilised with 10% iodine solution. Different sets of sterile surgical instruments were used to cut down through the skin, the thoracic and abdominal walls, and to remove the lungs and heart from the thoracic cavity and the spleen from the peritoneal cavity. Macroscopic evidence of TB infection was sought

by careful inspection, palpation and dissection of lungs, broncho-hilar and para-tracheal lymph nodes, spleen, liver, kidneys, adrenal glands, and mesenteric and porta hepatis lymph nodes.

Autopsy results were graded according to the following:

- **Normal** = No abnormal findings detected.

- **Non-specific** = Abnormal but non-specific changes (for example: pneumonia or pulmonary congestion, pleurisy, pericarditis, pericardial effusion, splenomegaly).

- **Tuberculosis** = Macroscopic evidence of tuberculosis: 2-5 mm diameter hard, white primary foci located in the lung tissue, usually in the peripheries but sometimes deep in the lung parenchyma. Associated enlarged, hard broncho-hilar or para-tracheal lymph nodes, often with evidence of caseous necrosis on section. Sometimes secondary granulomas in spleen, liver or primary lesion free lobes of the lung.

3.2.11 Culture of guinea pig organs

Lungs, broncho-hilar and para-tracheal lymph nodes and spleens were routinely harvested for TB culture, in addition to any suspicious lesions in other organs. When macroscopic evidence of TB was detected, lesions were divided, and half processed for TB culture, the other half being stored in 10% formaldehyde. When no evidence of TB was detected, half of the spleen and broncho-hilar and para-tracheal lymph nodes were cultured for tuberculosis and also a piece of lung approximately a quarter of the

size of a diaphragmatic lobe. Abnormal lung tissue, if present, was selected. Homogenisation was performed by hand in glass homogenisers, with 2 ml of sterile 0.9% saline solution. Tissue homogenates were decontaminated for 15 minutes using the NaOH-NALC method (0.5% NaOH and 1% N-acetyl-L-cysteine-sodium hydroxide (NALC)).[162] Following decontamination the resulting mixture was centrifuged at 3000 rpm for 15 minutes at 17 ^{0}C and the pellet re-suspended in 2 ml of 0.9% saline plus 0.2% bovine serum albumin (Sigma Chemical Co., St Louis, Mo., USA). 125 µl of the re-suspended decontaminated tissue homogenate was added to each of two wells containing 1.125 ml Middlebrook 7H9 broth medium (Difco^{TM}, Detroit, Michigan, USA) supplemented with oleic-acid, albumin, dextrose, and catalase (OADC; Remel, Lenexa, Kansas, USA) and an antimicrobial supplement PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin; Beckton, Dickinson & Co., New Jersey, USA)[163] and also to standard Lowenstein Jensen slopes for culture of *Mycobacterium tuberculosis*.[164] Positive cultures were sub-cultured on plates containing 7H11 medium (Difco^{TM}) for subsequent drug sensitivity testing, DNA fingerprinting and cryopreservation at -70 ^{0}C. Drug sensitivity testing on *M. tuberculosis* isolates was performed as part of the routine laboratory service of drug sensitivity testing using the Tetrazolium Microplate Assay.[165]

3.3 Results

3.3.1 PPD skin test responses in quarantine guinea pigs

Two batches of guinea pigs were transferred to the exposure chamber after quarantine, 144 in May 2002 and 148 in November 2002. The first batch of animals had three separate monthly PPD tests in quarantine; the second batch had two monthly tests. During the entire quarantine period in 2002, 760 PPD skin tests were performed on a total of 308 animals. 12 animals died in quarantine or following transportation to Lima. Figure 3.3 demonstrates the frequency distribution of these skin tests by PPD reaction size, measured as diameter of induration (mm) at 48 hours. Mean reaction size was 3.6 mm (SD 1.2).

It is noticeable that there are less half millimetre readings. This arose following analysis of the first month of skin tests (seen in Figure 3.10) where reduced totals for half mm reaction size values were noticed. This was felt to be due to an unconscious inclination by the reader to measure to the nearest millimetre. Thus in reading the third skin test in quarantine for the first batch of animals and the first skin test for the second batch, reaction sizes were rounded to the nearest integer, and this accounts for the reduced totals for half millimetre reaction size values seen in the histogram in Figure 3.3. However, it subsequently became apparent that a half millimetre resolution would be more useful for the determination of the cut-off point between a negative and positive test, and the original half millimetre readings were resumed for the final quarantine skin test. It is also noticeable in Figure 3.10 that there is an unusual peak at 5 mm for the month 2 skin test readings that has been verified from the original skin test notes. This was possibly due to an inclination by a reader to overestimate 5 mm as

an average negative reading (veterinary staff assisted with quarantine skin test readings). Practices were changed in light of this error, and it was decided to use the same reader (the PhD thesis author) for future experiments. If only the 453 quarantine skin tests read to half millimetre resolution are analysed, mean reaction size was 3.7 mm (SD 1.3), and these quarantine results to not impact on overall conclusions.

3.3.2 PPD skin test responses in unexposed negative control guinea pigs

The unexposed negative control group located on the hospital roof breathing ambient air initially comprised 14 animals, increasing to 50 over a total period of 13 months. In total, 287 PPD skin tests were performed in this group, and the results are shown in Figure 3.4. Mean induration at 48 hours was 4.0 mm (SD 1.5).

3.3.3 PPD skin test responses in guinea pigs exposed to ward air

A total of 292 animals were transferred to the exposure chamber, 144 in May 2002 and a further 148 six months later. Guinea pig air sampling continued until October 2003. The number of guinea pigs breathing ward air each month in the exposure chamber is shown in Figure 3.5. The attrition of animal numbers was due to animals being removed for sacrifice and autopsy following a large PPD skin test (>7 mm) or as randomly selected exposed PPD negative controls. Further attrition of numbers was due to intercurrent deaths, of which there were 25 over the whole period. After six months a new batch of animals was added to the chamber, resulting in an increase in numbers exposed in month seven to 204.

16 serial skin tests were performed at monthly intervals. This generated 1564 PPD skin test results. Figure 3.6 shows the frequency distribution of the reaction sizes of

these skin tests measured as induration at 48 hours (mm). An example of a small PPD skin test reaction of 4 mm diameter of induration is shown in the photograph in Figure 3.7, and a large PPD skin test reaction of 28 mm diameter of induration is shown in Figure 3.8. The percentage of animals tested each month with PPD skin test reactions of 7 mm; ≥7.5 mm <14 mm; and ≥ 14mm is shown in Figure 3.9.

3.3.4 Effect of serial PPD skin testing on PPD reaction size

The possible effect of serial PPD skin tests on PPD reaction size was investigated by consideration of the three monthly quarantine skin tests for the first batch of animals, and the two successive quarantine skin tests for the second batch of animals. Three histograms showing the reaction sizes for the first group are shown in Figure 3.10, and two histograms showing the reaction sizes for the second group are shown in Figure 3.11. In the first group, 148 tests in the first month resulted in mean reaction size 4.0 mm (SD 1.1); 147 tests in the second month resulted in mean reaction size 3.7 mm (SD 1.4); and 147 test in the third month resulted in mean reaction size 3.6 mm (SD 1.3). A paired t-test on the results for 147 animals showed no significant difference between month one and month two ($p=0.06$) or between month two and month three ($p=0.7$). Between month one and month three there was a significant decrease in mean reaction size of 0.4 mm ($p=0.01$), but this difference was biologically of minimal significance. In the second group, 160 tests in the first month resulted in mean reaction size 3.4 mm (SD 1.4) and 158 tests in the second month mean reaction size 3.6 mm (SD 1.4). A paired t-test for 158 test results showed no significant difference between these groups ($p=0.3$).

3.3.5 Male *vs.* female guinea pigs

Half of the first batch of 144 animals transferred to the exposure chamber was female. Of the second batch of 148, 60 (41%) were female and 88 (59%) were male. There was a high preponderance of fighting between male guinea pigs, despite animal density in cages being within accepted guidelines. This resulted in wounds and subsequent scarring on the dorsal aspect of male animals, and occasional deaths. It was thus proposed to use only female animals in future studies, and PPD skin test responses in males and females were therefore compared.

There was no significant difference between PPD skin test responses in males and females in quarantine (un-paired t test; $p=0.2$). However, in exposed animals there was a small but significant difference between the PPD reaction sizes of males and females (Mann-Whitney U test; $p=0.001$). The frequency distributions of PPD skin test reaction sizes (induration at 48 hours) in male and female exposed guinea pigs are shown in Figures 3.12 and 3.13. Median diameter of induration at 48 hours was 4.5 mm in females and 4.0 mm in males. Despite this small difference, however, there was no significant difference between the rates of PPD skin tests $\geq$7 mm in male and female animals (chi-square test; $p=0.8$). In all exposed males, 92 of 841 (11%) of tests were $\geq$7 mm compared with 83 of 723 (11%) of tests in all exposed females. Considering skin tests $\geq$7.5 mm, in all exposed males, 88 of 841 tests (10%) were $\geq$7.5 mm compared with 72 of 723 tests (10%) in all exposed females. There was no significant difference between the rates of PPD skin tests $\geq$7.5 mm in male and female animals (chi-square test; $p=0.7$).

3.3.6 PPD skin tests in unexposed '*M. bovis* positive control' guinea pigs

Thirteen '*M. bovis* PPD positive control' animals were maintained in the negative control animal enclosure, breathing unfiltered outside air. All animals challenged with the *Mycobacterium bovis* sensitising agent developed large skin test responses ≥7.5 mm. Median reaction size for 93 skin tests conducted within 6 months a sensitising injection was 16 mm (inter-quartile range 8.5-20 mm). Some animals demonstrated small reactions <7.5 mm several months after the initial injection with *Mycobacterium bovis* sensitising agent. In all of these animals large reactions were again observed following re-challenge with the sensitising agent. Large skin test reactions were seen in the *M. bovis* positive control animals at every skin test, providing evidence that the batches of diluted PPD used on each occasion had satisfactory biological activity.

3.3.7 Autopsies on guinea pigs exposed to ward air

A total of 267 autopsies were performed on ward air exposed guinea pigs that were sacrificed following a PPD skin test. Culture of homogenised organs for TB was carried out on 264 of these animals. A further 25 autopsies were performed on intercurrent deaths, i.e. animals that died in the exposure chamber in between monthly skin tests. Culture of organs for TB was performed for 21 of these animals.

Evidence of TB acquired by the airborne route was considered as a macroscopic primary focus in the lung with associated enlarged, hard broncho-hilar or para-tracheal lymph nodes, with or without evidence of caseation. Caseation was denoted by the presence of a soft, creamy white substance on section of a lymph node, sometimes requiring the application of light pressure to become evident. Figure 3.14 demonstrates the thoracic cavity of a guinea pig with a primary focus of TB in the lateral lobe of the

left lung. Figure 3.15 demonstrates a large para-tracheal lymph node which has been sectioned to show macroscopic evidence of caseous necrosis. Figure 3.16 shows a guinea pig spleen in situ with multiple pale coloured tuberculous nodules.

Evidence of TB was seen in a total of 134 animals. Lung primary foci were observed in 130 of these. In the 4 (2%) animals without macroscopic evidence of a primary focus in the lung, enlarged, hard, caseous broncho-hilar lymph nodes were seen. This was also considered sufficient evidence for airborne TB infection, in accordance with the observations of Riley and Lurie.[75,76,158] In the four years of Riley's study, primary lung foci were observed in 104 (78%) of all guinea pigs with proven TB.[75,76] In 34 animals in this study multiple primary foci were seen in the lungs. Four separate foci were seen in one animal, three separate foci were seen in nine animals and two were seen in 24 animals. These are likely to represent multiple 'hits' with droplet nuclei and the possibility of infection with multiple strains is discussed in the next chapter. Of the total 175 primary foci observed, 103 (59%) were found in diaphragmatic lung lobes, 35 (20%) in lateral lobes, 24 (14%) in cardiac lobes and 13 (7.4%) in apical lobes. This distribution mirrors the approximate differences in the volumes of these lung lobes and presumably their approximate ventilation (anatomically the diaphragmatic lobes are the largest and the apical lobes smallest). The majority of primary foci were superficially located, evident on the surfaces of lung lobes, and only 12 (7%) were located deep in the lung parenchyma, detected at autopsy by palpation.

Figure 3.17 demonstrates the results of autopsies according to PPD reaction size for the 267 sacrificed animals that had been exposed to ward air. For the 95 animals with PPD skin test reactions <7 mm there was macroscopic evidence of TB in only 3 animals (3.2%); for the group with a 7 mm reaction there was evidence of TB in 4

animals (31%); for animals with reactions 7.5-13.5 mm there was evidence of TB in 49 animals (64%) and for reactions ≥14 mm there was evidence of TB in 74 animals (89%). Also shown in Figure 3.17 are the results for autopsies performed on the 25 intercurrent deaths, of which 4 (16%) had evidence of TB infection.

3.3.8 Autopsies of unexposed negative control guinea pigs

A further 35 autopsies were performed, on unexposed negative control animals, i.e. animals that breathed ambient roof air only. There was no macroscopic evidence of tuberculosis detected in any of these animals. 20 autopsies were completely normal and the remainder had non-specific changes.

Autopsies were not performed blinded to experimental group or to PPD reaction size, but all cultures were performed blinded to the source of organs. For future experiments, this oversight was corrected and all autopsies are being performed blinded both to experimental group and PPD reaction size.

3.3.9 Culture of guinea pig organs

Organs were harvested from 267 autopsies of animals exposed to ward air that were sacrificed following a PPD skin test. TB cultures were carried out on homogenised organs for 264 of these animals. Autopsies with TB cultures were carried out on 21 of the 25 animals that were intercurrent deaths, making culture results available for a total of 285 animals. Of these, 135 animals were culture positive in at least one tissue. These cultured tissues comprised 183 broncho-hilar or para-tracheal lymph node specimens, 179 pulmonary specimens, 223 spleen specimens, 99 combined pulmonary and broncho-hilar or para-tracheal lymph nodes specimens, 47 liver specimens, and 21

specimens of other tissues such as additional primary lung foci or abnormal mesenteric lymph nodes. When only considering the 135 animals culture positive in at least one tissue, for hilar or para-tracheal lymph nodes, 52 (93%) of 56 specimens were positive; for lung specimens 44 (81%) of 54 specimens were positive; for spleen 58 (61%) of 95 specimens were positive, and for the combined lung and lymph node specimens 70 (99%) of 71 specimens were positive. None of 47 liver specimens were culture positive, and six of the specimens of other tissues were positive. Five of these were additional primary lung foci, and one was a mesenteric lymph node in an animal that shared a non-drop-through bottomed cage for several months with another tuberculous guinea pig whilst awaiting autopsy.

3.3.10 Culture results in relation to PPD reaction size

Figure 3.18 shows the number of culture positive guinea pigs (i.e. a positive culture for *M. tuberculosis* in at least one animal tissue) according to PPD skin test reaction size, for a total of 264 animals. Emphasis is given to reaction sizes around 7.5 mm, a putative cut-off between a positive and a negative test. Figure 3.19 is a similar figure, but includes additional results derived from the autopsies. Specifically, four guinea pigs with PPD skin test diameters of 7, 10, 10.5 and 11 mm were culture negative, but had autopsies with diagnostic evidence for TB. These animals are re-classed as 'positive' in Figure 3.19. Two guinea pigs with PPD skin test diameters of 12 mm had no cultures, but had autopsies with diagnostic evidence for TB, and another animal not cultured had a skin test diameter of 7 mm but a normal autopsy. These animals were also added to Figure 3.19, making a total of 267 animals for which a PPD skin test can be coupled to a diagnosis for TB based on either autopsy or culture.

3.3.11 Culture results in relation to autopsy results for guinea pigs likely to have tuberculosis

In guinea pigs with large skin test responses ≥7.0 mm, 169 pairs of autopsy and culture results were available. For 125 autopsies with macroscopic evidence of TB, 121 (97 %) were also positive in culture. For 40 autopsies with non-specific changes, only 6 (15%) were positive in culture, and all of only four autopsies with no abnormal findings were also culture negative. These data are presented graphically in Figure 3.20.

3.3.12 Determination of the cut-off for a positive PPD skin test

The data shown above in Figure 3.19 has been used to calculate sensitivities and specificities for this guinea pig PPD skin test for cut-off points from ≥5.5 mm up to ≥14 mm and this is presented in Figure 3.21 as a ROC curve for this test (Receiver Operating Characteristic Curve).[166] The area under this curve is 0.91, suggesting that the accuracy of this test is excellent.[166] Putative cut-off points of ≥7 mm and ≥7.5 mm were considered most suitable. By consideration of Figure 3.3, the PPD skin test responses in quarantine animals, a cut-off point of ≥7 mm would include 6 (0.8 %) of 746 readings as positive. A cut-off point of ≥7.5 mm would not include as positive any animals in this group. By consideration of Figure 3.4, the PPD skin test responses in unexposed negative controls breathing ambient roof air, a cut-off point of ≥7 mm would include 4 (1.4 %) of 287 readings as positive. A cut-off point of ≥7.5 mm would not include any animals in this group as positive. It can be seen from Figure 3.19 that four of the 13 animals with a reaction size of 7 mm were positive for TB at autopsy or in culture. One of these animals was sacrificed shortly after this skin test of

7 mm. The other three, however, were kept alive to monitor the PPD response over time. The first had a further four monthly skin tests, with a peak reaction diameter of 9 mm. The second had a further five monthly skin tests, with a peak reaction diameter of 20 mm, and the third had a further seven monthly tests, with a peak reaction diameter of 24 mm.

In light of the results presented above, in order to minimise guinea pig deaths, a cut-off point of ≥7.5 mm was selected for a positive PPD skin test using 100 Units of PPD tuberculin in these Peruvian guinea pigs exposed to natural aerosols of TB generated by TB patients.

3.3.13 PPD skin test readings at 24 *vs.* 48 hours

Riley's original guinea pig air sampling study measured PPD skin test responses at 48 hours, as is normal practice in humans. For the first 15 months of this study, 48 hour measurements were made. Following discussion with an expert in the guinea pig model of TB who suggested that readings at 24 hours had greater sensitivity (David McMurray, Texas A&M University, USA) it was decided to compare readings at 24 and 48 hours. A group of 604 animals in quarantine awaiting further experiments had two monthly skin test responses measured at 24 and 48 hours for comparison. These animals were from the same breeder as the animals in the exposure chamber, and maintained under the same conditions as they had been, in the same quarantine facility. Two further groups of animals had PPD skin tests read at 24 and 48 hours for this analysis. The first group comprised 20 PPD positive guinea pigs presumed to be infected with TB that had been removed from the exposure chamber in study month 10 following a large TB outbreak, and were maintained in isolation awaiting sacrifice and

autopsy. The second group comprised 10 PPD positive '*M. bovis* positive controls' (the animals previously injected with *Mycobacterium bovis* sensitising agent).

Figure 3.22 demonstrates results for all skin tests read at both 24 and 48 hours. These comprised 917 skin test readings in 604 un-exposed animals in quarantine over two monthly skin tests and 91 skin tests in the 'PPD positive' animals i.e. 10 '*M. bovis* positive controls' and 20 animals with presumed tuberculosis awaiting sacrifice. It can be seen from the scatter of points to the left of the line in Figure 3.22 that there was a group of skin tests readings that were larger at 24 hours compared with 48 hours. For the quarantine group, mean skin test diameter at 24 hours was 3.7 mm (SD 1.31) and at 48 hours was 3.7 mm (SD 1.21) and there was no significant difference between these two groups of readings (paired t-test; $p=0.8$). For the 'PPD positive' group, median skin test diameter at 24 hours was 10 mm (inter-quartile range 5.5-16 mm) and at 48 hours was 7 mm (inter-quartile range 5-10.5 mm) and there was a significant difference between these two groups of readings (Wilcoxon Signed Ranks Test; $p<0.001$). Figure 3.23 demonstrates how in the presumed PPD positive group of animals, at 24 hours 54 (59%) skin tests would have been classed as positive, and at 48 hours only 41 skin tests (45%) would have been classed as positive using ≥ 7.5 mm as the cut-off for a positive PPD skin test ($p=0.05$; chi-square test).

3.3.14 Effect of total exposure duration on the likelihood of developing a positive PPD skin test

It was postulated that there may be an accumulation in the exposure chamber over time of animals resistant to TB infection due to the repeated removal, every month, of PPD positive and therefore susceptible animals. The first cohort of animals, that

entered the chamber in May 2002, had already been exposed for 6 months when the second batch began exposure to ward air in November 2002. The PPD skin test responses of animals in the first group were thus compared to the PPD skin test responses of those in the second for the exposure period from November 2002 onwards. In the first group, 38 (14 %) of 265 tests were classed as positive (≥7.5 mm) compared with 95 (14 %) of 671 tests in the second group. There was no significant difference in the rate of PPD skin tests ≥7.5 mm between the first group and the second group (p=0.9; chi-square test).

3.3.15 Guinea pigs with false negative PPD skin tests

Of the 267 animals with a PPD skin test result and a corresponding autopsy or culture diagnosis for TB, 95 had reactions <7 mm, and 13 had reactions of 7 mm, as seen previously in Figure 3.19. Five of these <7 mm skin test reactors had TB on culture of organs, and four of the 7 mm skin test reactors had TB on autopsy or culture of organs. Thus with a cut-off PPD value of ≥7.5 mm, there would be a false negative rate of 8.3%, and if considering the 7 mm reactors as 'borderline', the false negative rate in the <7 mm skin test reactors would be 5.3%.

Of the five false negatives with skin tests <7 mm, all had separate cultures of three tissues (broncho-hilar lymph nodes, lung and spleen). Three had autopsies diagnostic for TB, of which two were culture positive in lymph nodes, lung and spleen, and the third positive in both lymph nodes and lung but negative in spleen. One of these animals had gross weight loss.

Of the remaining two animals, one had an abnormal but non-specific autopsy and was positive in spleen only. The final animal had a normal autopsy, and was culture positive in broncho-hilar lymph nodes only. DNA fingerprinting of these TB strains was the same as some of the patients on the ward (see next chapter).

3.4 Discussion

The results presented in this chapter demonstrate the successful recreation of the classic studies on tuberculosis transmission conducted by Riley and colleagues in the late 1950s and early 1960s.[75,76] This guinea pig air sampling model for TB has been re-defined in this Peruvian setting in today's era of multidrug-resistance and HIV infection, to form the groundwork for further important studies of TB transmission and its control.

3.4.1 Determination of a positive PPD skin test

Much of the literature concerning tuberculosis infection in guinea pigs where tuberculin skin testing was employed relates to particular strains of guinea pigs, for example Hartley strains, that are specifically bred for scientific purposes and are guaranteed to be free of specific pathogens. There is no consensus on what represents a positive PPD skin test following tuberculosis infection, and in these animals, any PPD skin test reaction is often judged to be positive, representing mycobacterial infection. In addition, many investigators use a 5 Unit dose for the test, and if guinea pigs are infected by the airborne route, it is artificial aerosols to which the animals are exposed. Riley's work is the only published data on PPD responses in guinea pigs

naturally infected by the airborne route. As this research involves natural airborne infection, albeit from humans to animals, and has tried to emulate that of Riley, a dose of 100 Units was selected to correspond to that used in the original studies. It is not clear whether Riley used specific pathogen free animals, indeed it is uncertain they were available in the era of his study. In Riley's work, there were no PPD responses in quarantine animals greater than 5 mm. Riley used a response of 10 mm with an area of central necrosis to signify a positive test, but had identified a group of animals with intermediate sized PPD responses (6-13 mm in diameter without evidence of a central area of necrosis) of uncertain significance.[159] In this group of 64 animals, 24 were sacrificed and had autopsies performed, and six (25%) had autopsy evidence of tuberculosis. Further investigation of this group was not published as had been planned, perhaps owing to the sad death herself from tuberculosis of Cretyl Mills who had performed the work.[167]

The animals used in this study were not specifically pathogen free, and a diet of grass and alfalfa in the mountain breeding station of the veterinary school is likely to have exposed them to environmental mycobacteria. Such exposure could result in cross reactivity in the PPD skin tests,[168] especially using a dose as high as 100 Units. Furthermore, the water used in this study was not specifically sterilised, and though standard pellet feed was introduced for the animals in Lima, this was not treated in any way beforehand. With 760 PPD skin test results in quarantine for the first and second batches of animals prior to exposure to ward air, 287 results from unexposed negative control animals breathing ambient roof air, and a further 917 results in new quarantine animals for future experiments all being <7.5 mm, it is likely that a PPD reaction of 7.5 mm induration and above can be regarded as strong evidence of infection with TB,

irrespective of autopsy or culture findings. It is likely that animals with a 7 mm response represent the 'tail' of the frequency distribution of PPD responses in both tuberculous and non-tuberculous animals. Indeed 4 (31%) of 13 animals (see Figure 3.2) with a PPD response of 7 mm were positive for *M. tuberculosis* by culture or on autopsy evidence. The area under the ROC curve was 0.91, which is designated an 'excellent' test. The cut-off which gives the best trade off between sensitivity and specificity is generally considered to be the point on the curve that is nearest to the top left hand corner. The 7.5 mm cut-off is close to this point, and corresponds to a sensitivity of 93% and specificity of 77%. It is important to detect animals with TB so that they can be removed from the exposure chamber before they develop advanced disease which increases the risk of horizontal transmission between animals. For this reason, some loss of specificity is acceptable in selecting ≥7.5 mm as the cut-off point for a positive test. It is worth noting also that of the four animals with 7 mm skin tests who had TB on autopsy or culture, three of these animals went on to develop larger skin test responses on subsequent monthly testing as they awaited sacrifice and autopsy. It may be that they would have been negative at autopsy or on culture had they been sacrificed immediately after their initial 7 mm test result.

3.4.2 PPD skin test positive but autopsy and culture negative animals

The significance of PPD positive but autopsy and culture negative animals is interesting. Of 159 exposed guinea pigs with PPD responses greater than ≥7.5 mm, 30 (19%) were culture and/or autopsy negative. Guinea pig infection by the intra-muscular route with clinical strains of *M. tuberculosis* isolated from the sputum of patients has shown considerable differences in virulence for guinea pigs.[103,169-171] Similar differences in virulence have also been well described in guinea pigs infected

experimentally by the airborne route.[101,102,104,169] By six weeks, numbers of mycobacteria in the spleens or primary lung lesions of experimentally infected guinea pigs may fall below 100 colony forming units,[104] and it is possible that the primary focus may resolve over time. Owing to the monthly PPD system, and the approximate 21 day 'incubation' period in experimental guinea pigs from inhalation of a droplet nucleus which reaches the alveolar wall to the mycobacterial bacteraemia associated with PPD conversion,[172] an animal may have had an infection of between three and seven weeks duration at the time of the skin test. Thus if infection occurs with a strain of reduced virulence for guinea pigs, by the time of autopsy (sometimes delayed for logistical reasons) there may be little macroscopic evidence of TB, and the levels of mycobacteria may be below the detection limit of the autopsy-culture procedure used

Of the five false negative animals with small PPD skin test reactions <7 mm, three had autopsies diagnostic of TB, and were culture positive in more than one organ. It is likely that these represent true false negatives. Indeed the grossly underweight guinea pig in this group is likely to have developed anergy as a result of extensive disease. It is possible some animals had innate anergy, and this is supported by the presence of some zero PPD reactions in the quarantine, exposed and unexposed groups of guinea pigs. The remaining two false negatives may have represented cross-contamination in the laboratory given that their autopsies showed no evidence of TB, one being completely normal, and only being culture positive in one of three organs. The DNA spoligotype pattern (see next chapter) corresponded with that of patients who had been on the ward, however the laboratory processed specimens of many of these patients routinely. A further possibility is that these guinea pigs had early TB infection, prior to the bacteraemic phase which is thought to correspond to conversion to a PPD positive skin test.[172] Mycobacteria can usually be detected in guinea pig broncho-hilar lymph nodes by two weeks following aerosol infection, which is before the bacteraemic phase, and may precede PPD skin test conversion. This may explain the PPD false negative animal that was culture positive in broncho-hilar lymph nodes only. It is a less likely explanation for the animal that was culture positive in spleen, as a prerequisite for becoming TB culture positive in spleen is mycobacterial bacteraemia.

3.4.4 Implications of false negative PPD skin tests

The importance of animals with false negative PPD skin tests is that an animal may remain in the exposure chamber undetected for long periods and develop advanced disease. Lurie demonstrated tuberculosis transmission between guinea pigs by the respiratory and faecal-oral route, but this was over a period of many months.[158,173,174]

The danger therefore is of horizontal transmission inside the exposure chamber, which has the potential to confound studies of TB transmission using this model. As a result, drop-through cages were installed to prevent coprophagia and hence minimise the risk of horizontal transmission via the faecal oral route.

Poor quality PPD and operator error are other potential sources of false negative skin tests. Utmost care was taken in the preparation of PPD and maintaining it in the field at 4 ^{0}C, and in the positioning of each skin test to minimise operator error. Serial weighing of exposed guinea pigs was instituted to identify weight loss as a secondary indicator of TB disease.

The spatial distribution of guinea pig TB infection in the animal house was random in keeping with airborne infection from the ward. Further evidence for the absence of horizontal transmission between guinea pigs was provided by DNA fingerprinting analysis (see next chapter). For future work, the guinea pig exposure chamber has been completely re-designed to minimise the risk of horizontal spread. The new animal facility has a transverse instead of longitudinal direction of air flow, and cages of six guinea pigs are now effectively isolated from each other, in contrast to the original longitudinal design where guinea pigs 'downstream' in the airflow may contract TB from an infected animal 'upstream'. With the new design, a horizontal outbreak would be limited to one cage, which should be obvious by its geography and temporal association, corroborated by DNA fingerprinting.

3.4.5 Conclusion

In this chapter the results of a successful re-creation of classic studies on airborne tuberculosis transmission have been presented. The guinea pig model for the detection of natural airborne tuberculosis transmission has been re-validated, and defined for the type of guinea pigs commonly available in Peru. Through the use of this air sampling facility and refined guinea pig model, in the next chapter new insights into TB transmission in today's era of HIV infection are presented.

Figure 3-1: Airtight animal house on the roof of a TB ward

Air arrived on the roof via the two ducts at the extreme right of the picture, passed over the guinea pigs, and then passed through two extractor fans before being exhausted into the atmosphere through the two chimneys.

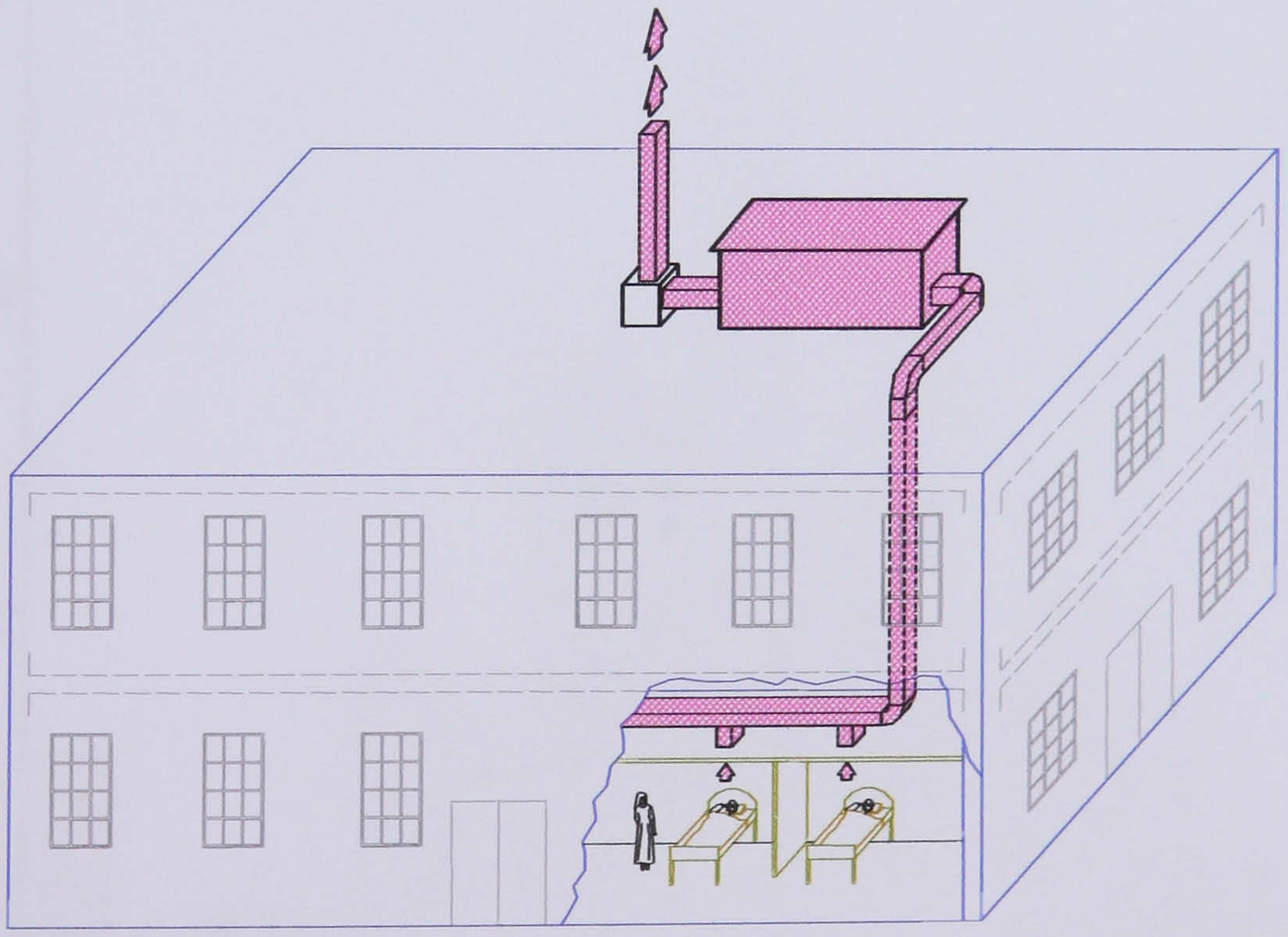


Figure 3-2: Schematic representation of animal house on the roof of a TB ward

The HIV-TB ward is located on the ground floor, and exhaust air flowed through ductwork in the false ceiling up to the animal house on the roof.

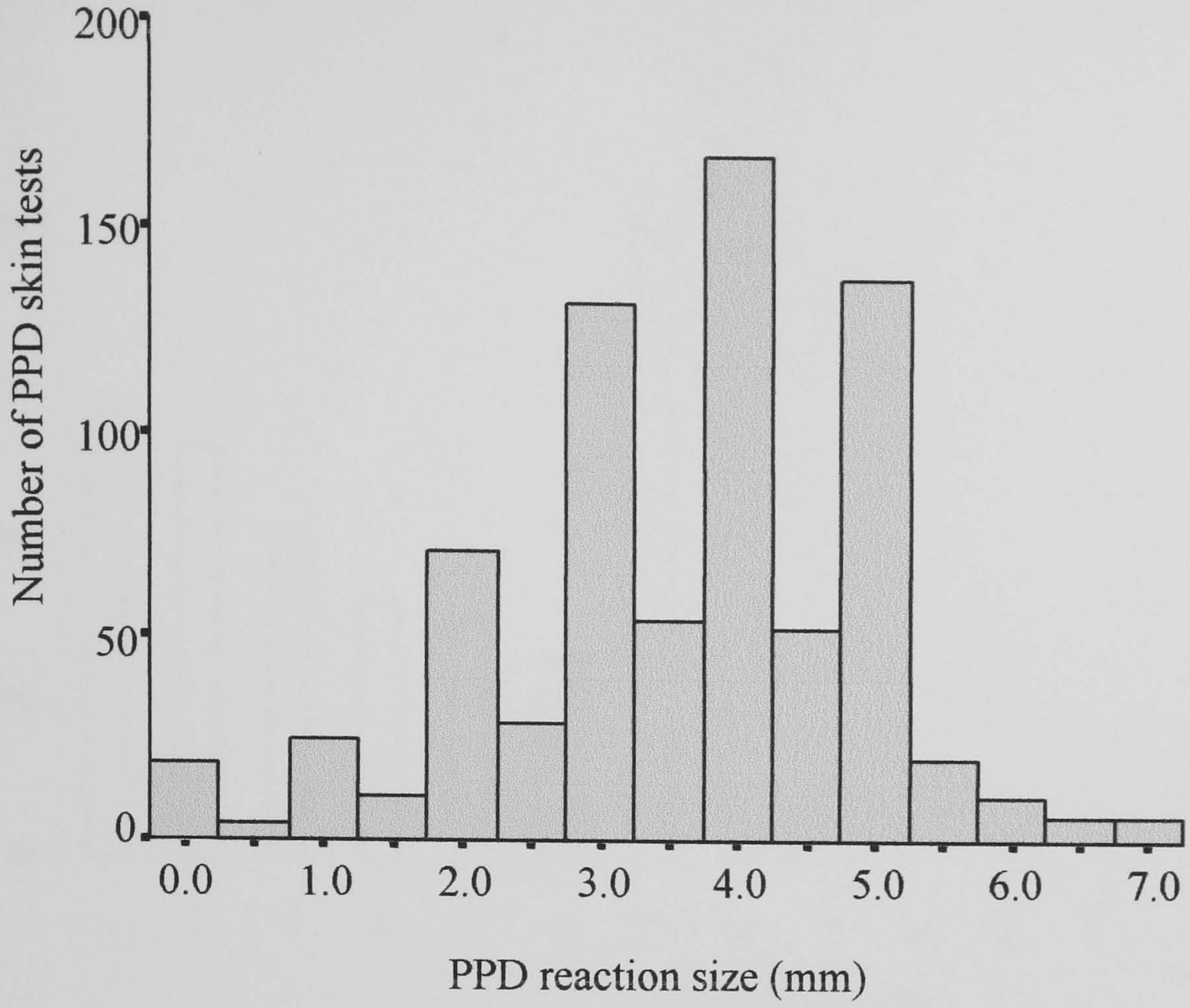


Figure 3-3: PPD skin test responses in unexposed guinea pigs in quarantine

760 tests were performed on a total of 308 animals over three months.

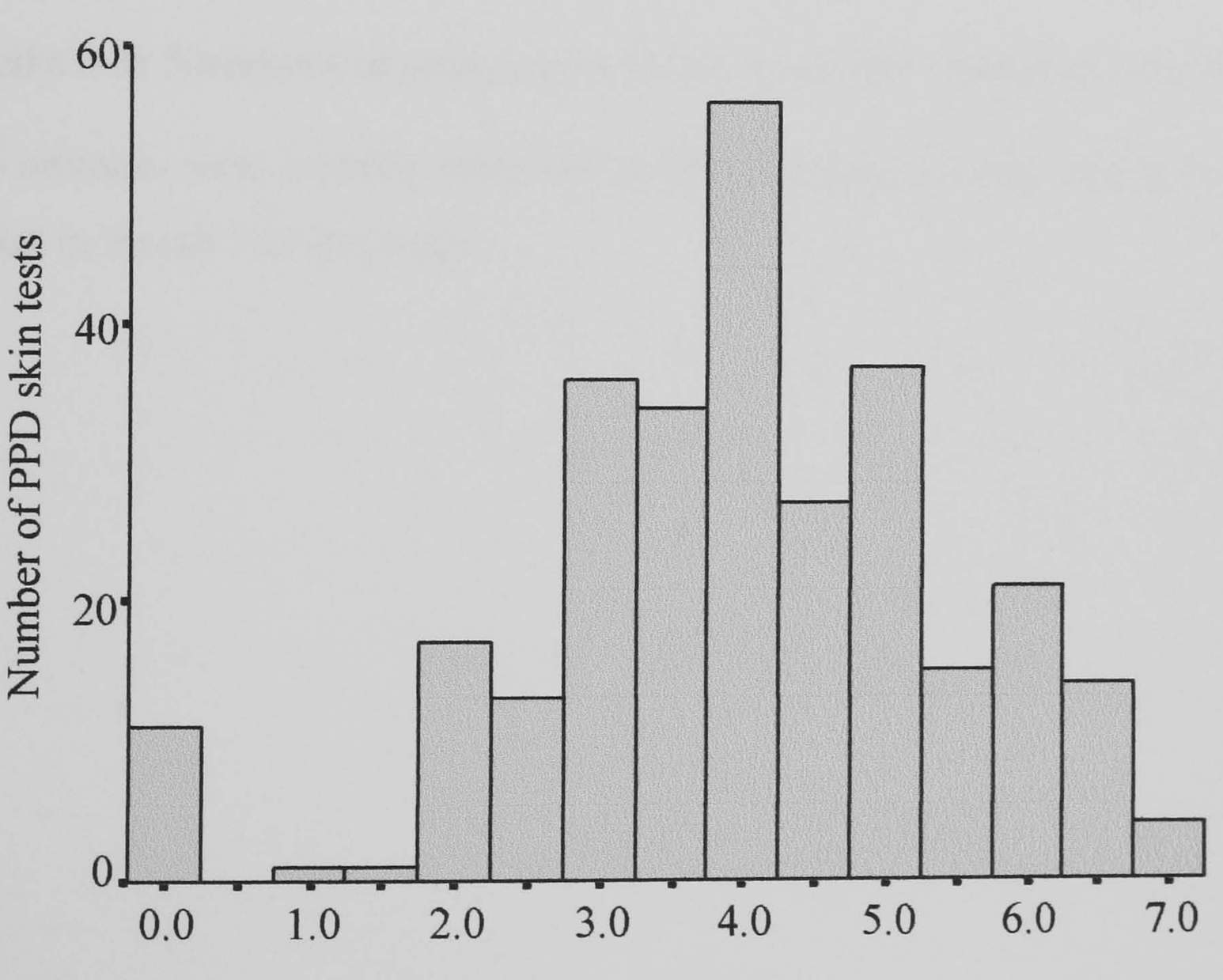


Figure 3-4: PPD skin test responses in unexposed negative control guinea pigs

287 tests were performed on a total of 50 animals over a period of 13 months.

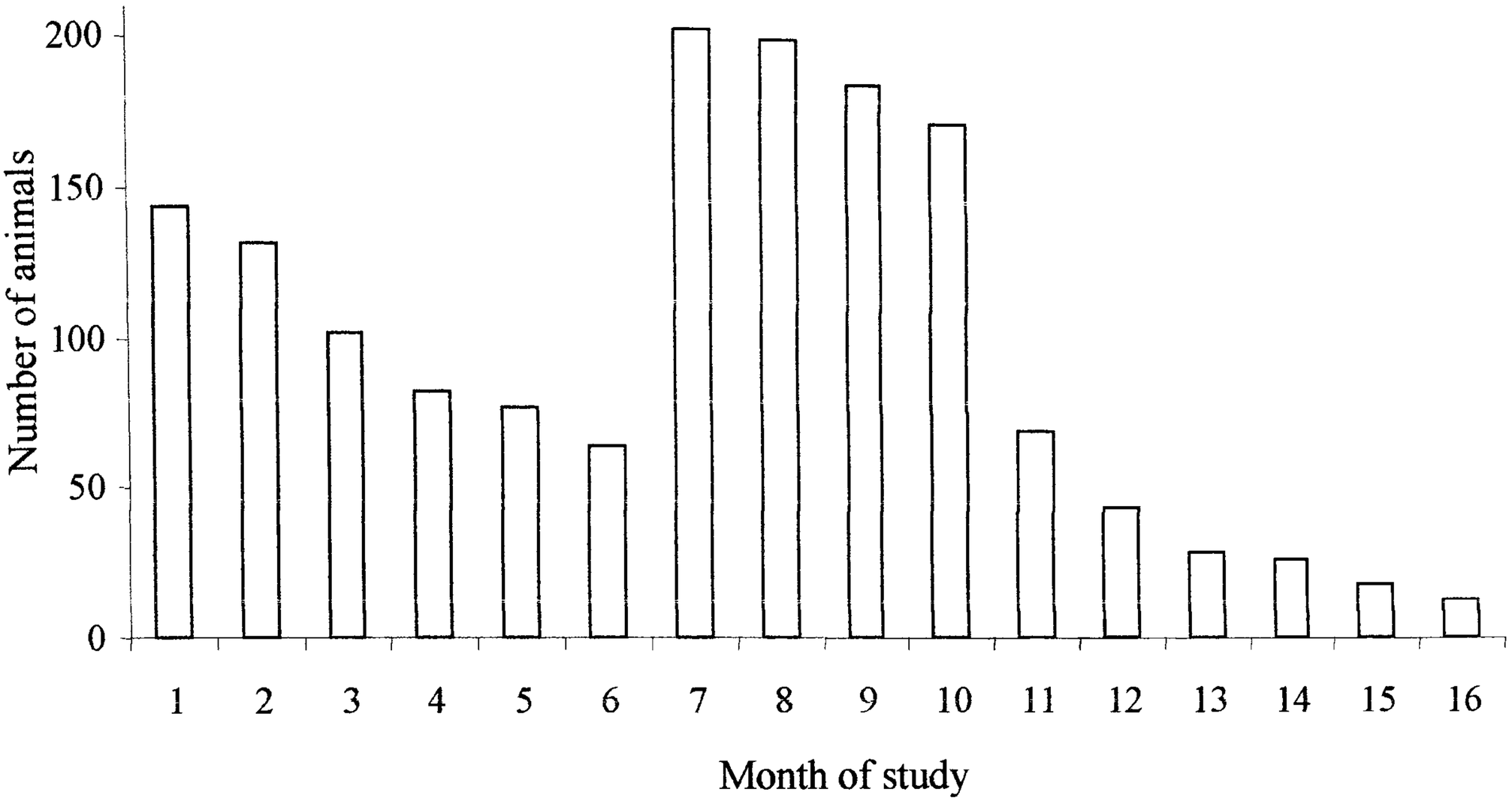


Figure 3-5: Numbers of guinea pigs in the exposure chamber breathing ward air

144 animals were initially installed in the exposure facility, and a further 148 were added in month 7 of the study.

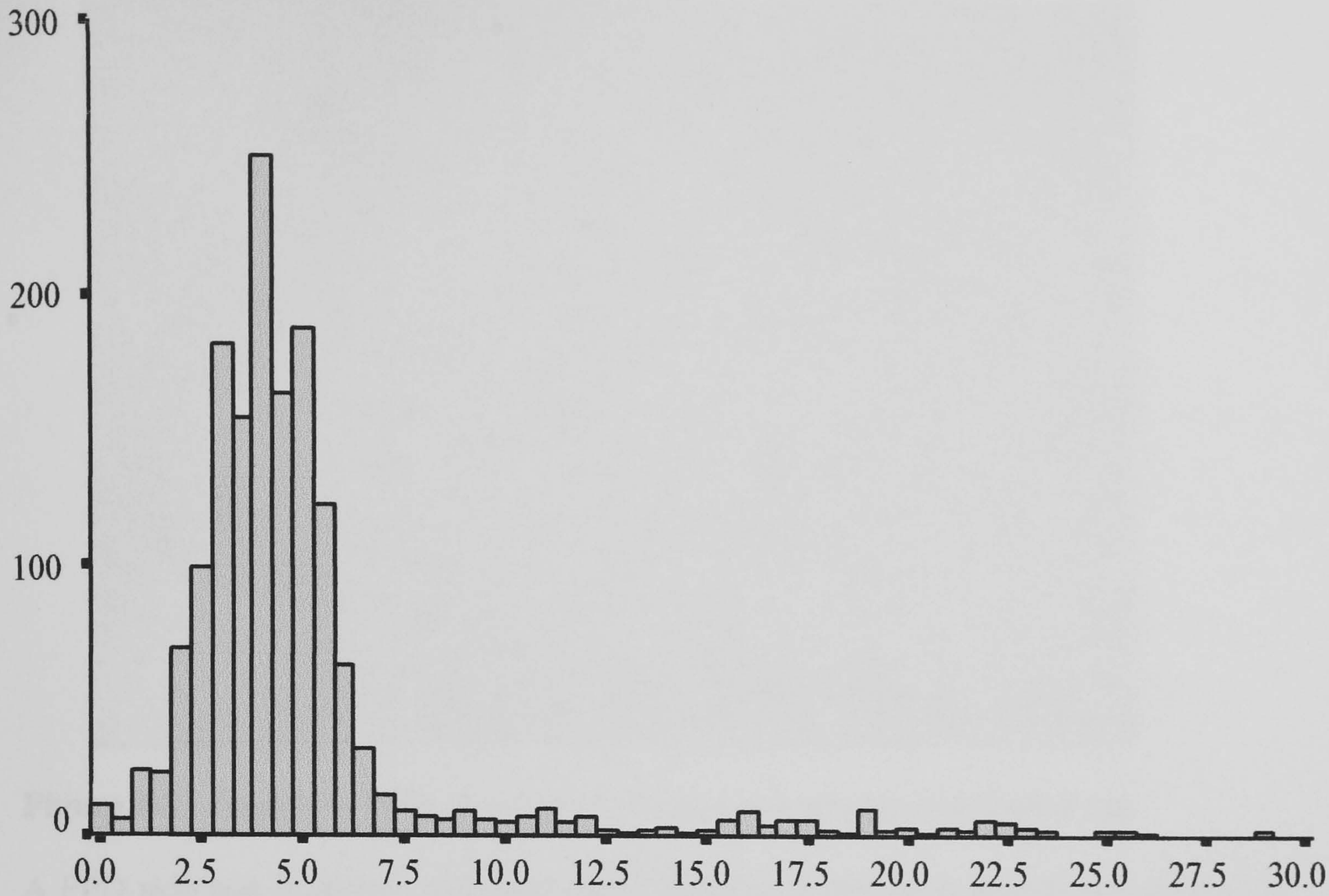


Figure 3-6: PPD skin test responses in all guinea pigs exposed to ward air

1564 tests were performed on a total of 292 animals exposed over a period of 16 months. The great majority were small reactions (between 0 and 7 mm) similar in size to those seen in the quarantine and unexposed groups, but there is also a group with large responses up to 30 mm in diameter, likely to represent exposure to airborne tuberculosis from the ward.

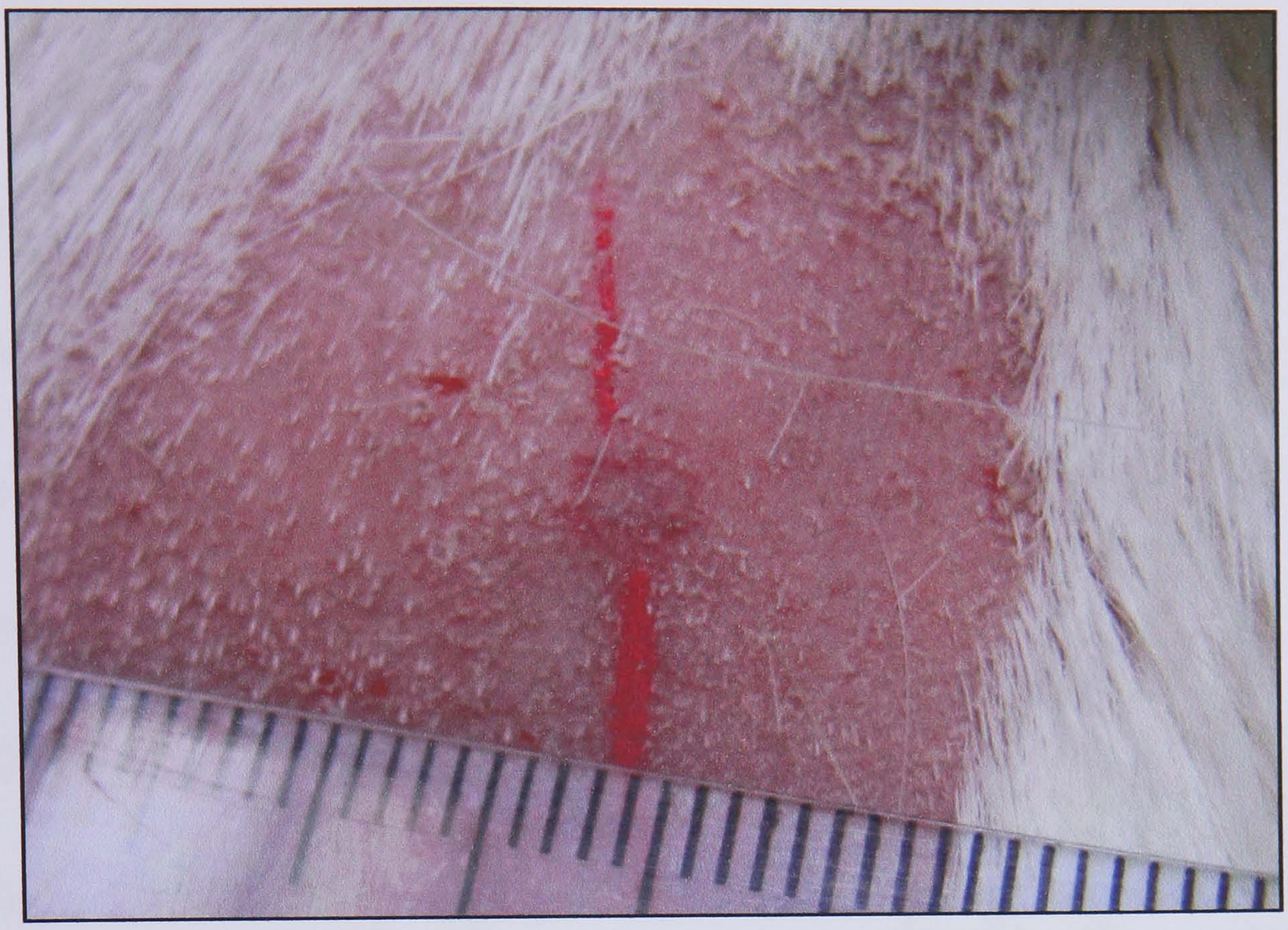


Figure 3-7: Negative PPD skin test reaction in an unexposed guinea pig

A PPD skin test is shown with induration diameter 4 mm in an unexposed guinea pig negative for tuberculosis. A red ball point pen has been used to demarcate induration.

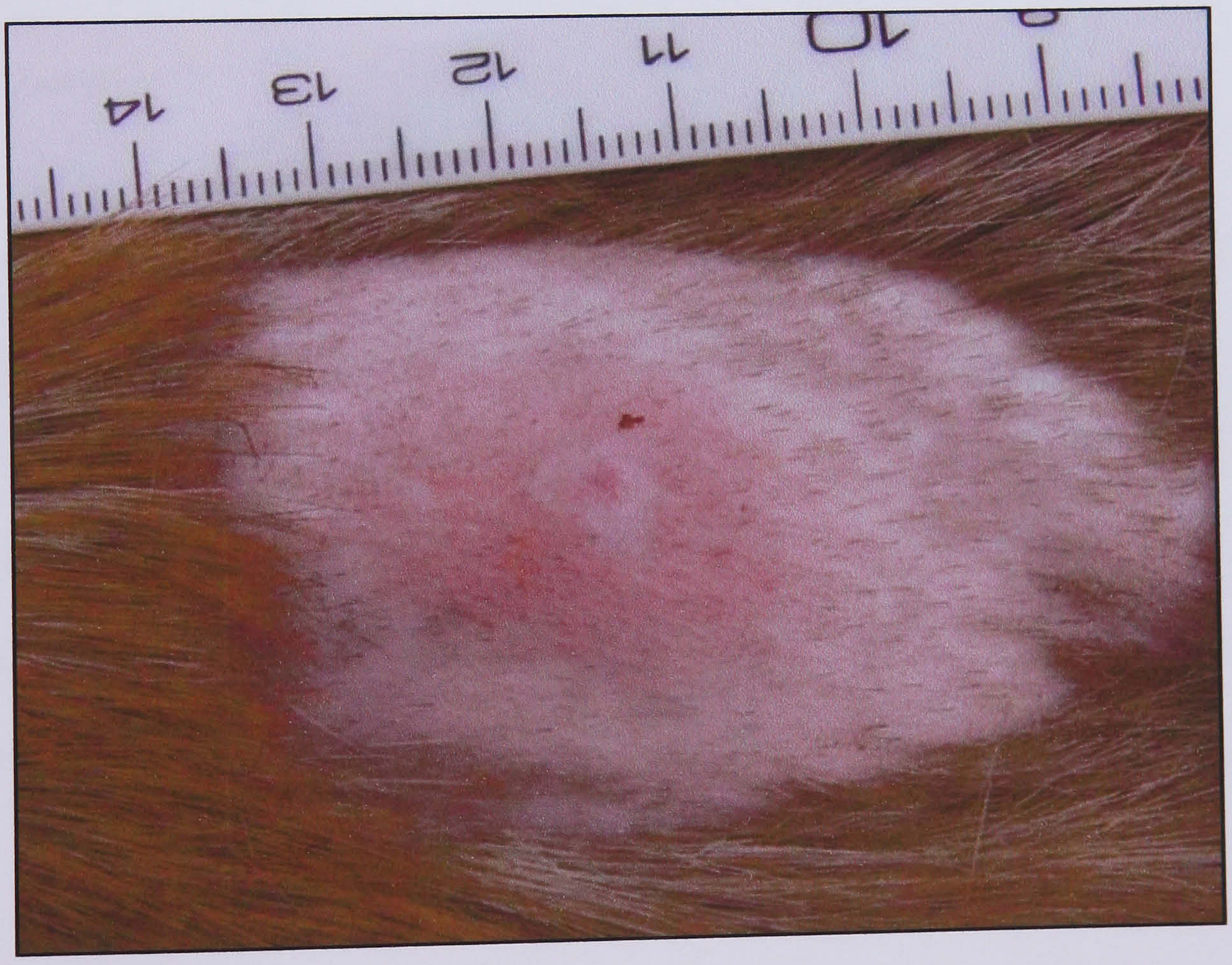


Figure 3-8: Positive PPD skin test reaction in a guinea pig with tuberculosis

PPD skin test with induration diameter 28 mm in a guinea pig with tuberculosis after spending 10 months in the exposure chamber breathing ward air.

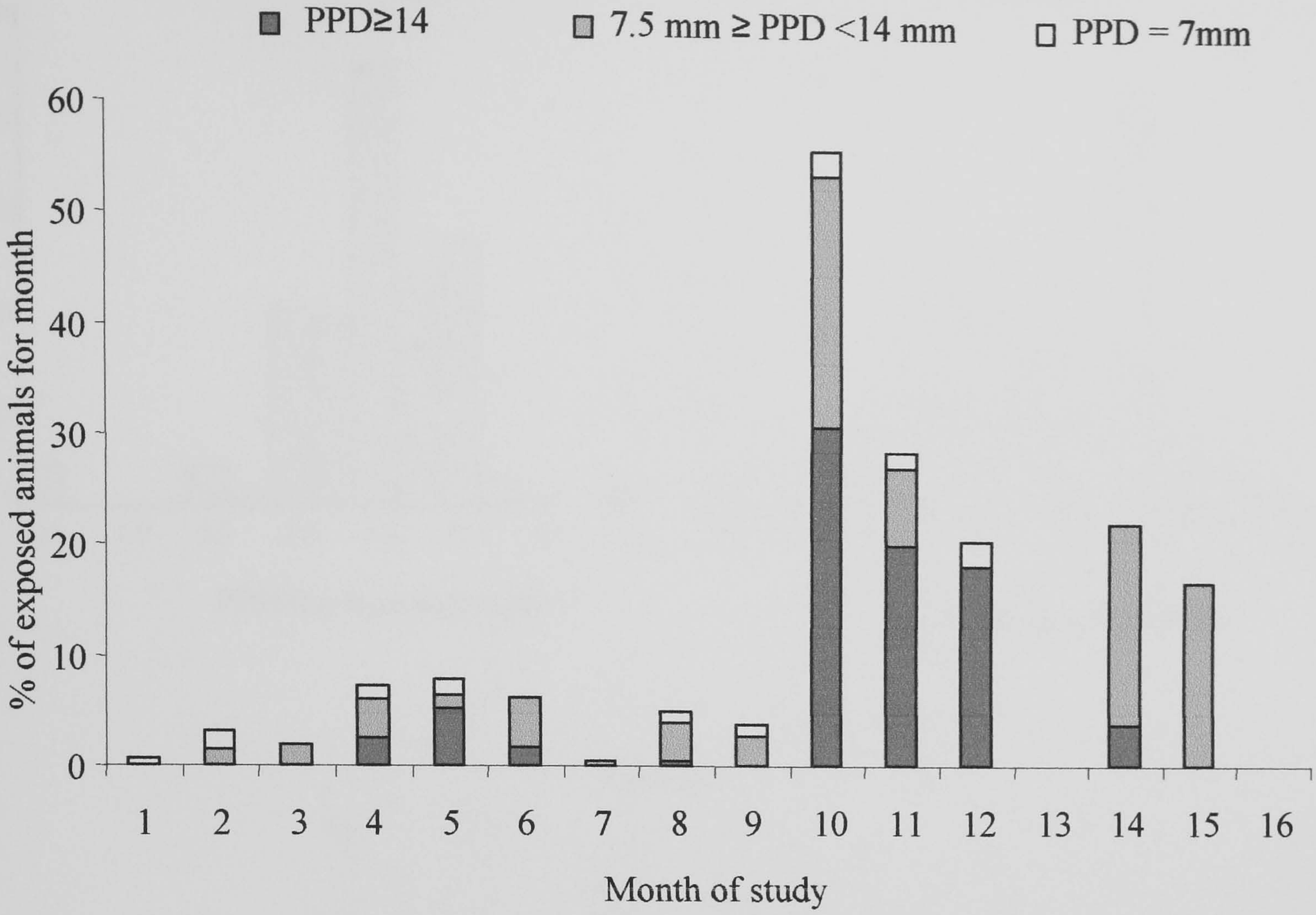


Figure 3-9: Percent of total skin tests each month with diameter of induration ≥7 mm for exposed guinea pigs breathing ward air

The dark shaded areas represent the percent of exposed animals for that month with large skin tests ≥14 mm, the light shaded areas the percent of exposed animals for that month with 'intermediate' skin tests of between 7 mm and 14 mm, and the un-shaded areas the animals with skin test diameters equal to 7 mm.

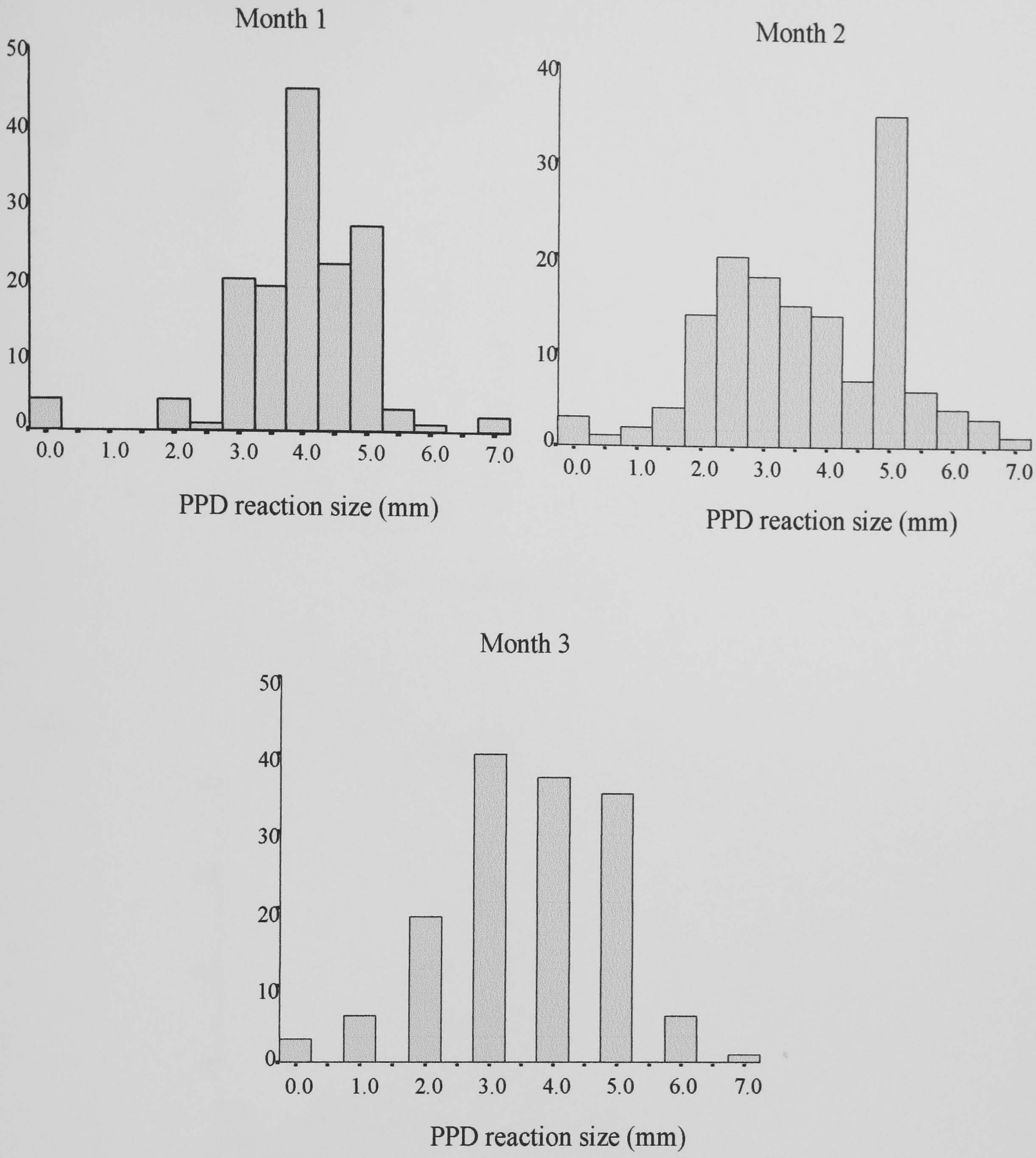
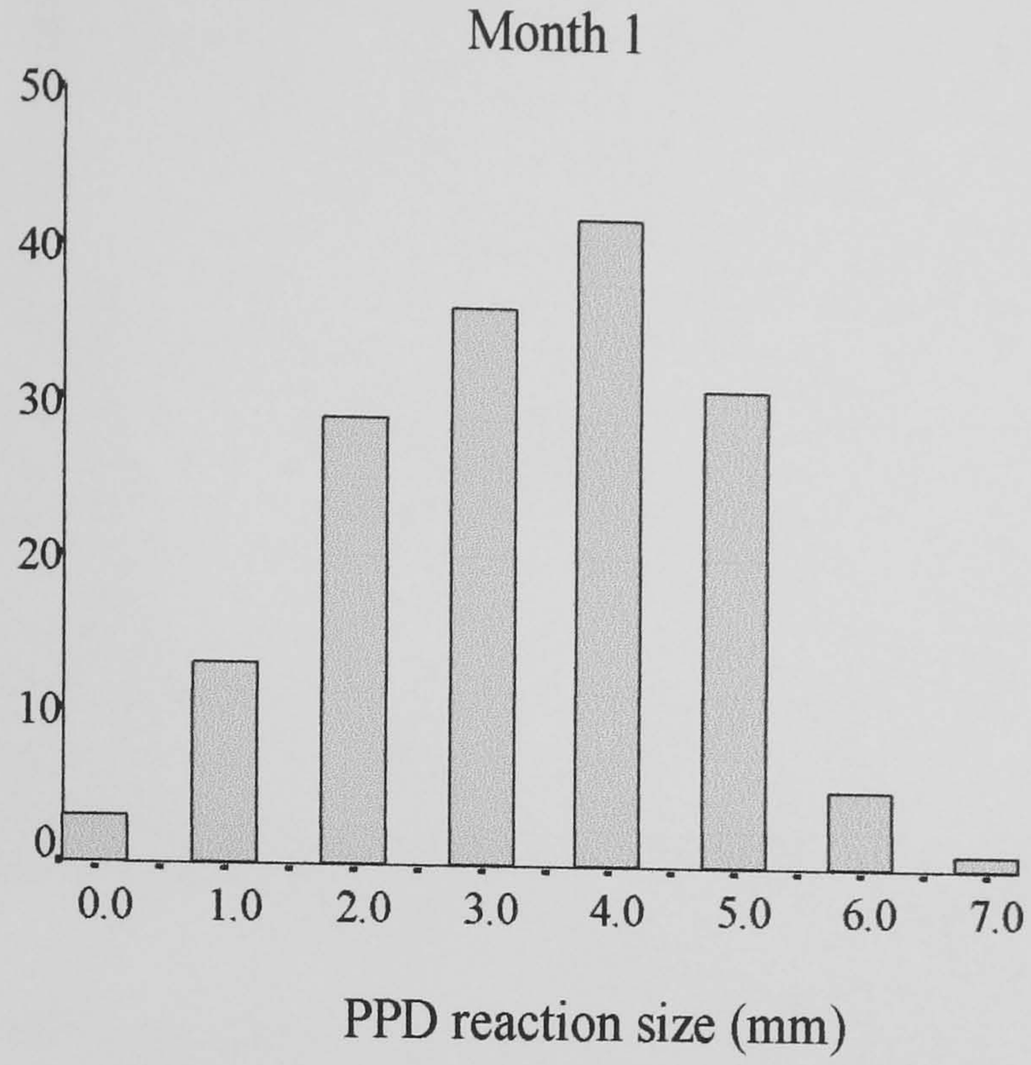


Figure 3-10: Serial skin test reactions for first group of animals over 3 month period in quarantine

For the first month, 148 tests resulted in mean induration diameter 4.0 mm. For the second month, 147 tests resulted in mean induration diameter 3.7 mm, and in the third month 147 tests resulted in mean induration diameter 3.6 mm.

(a)



(b)

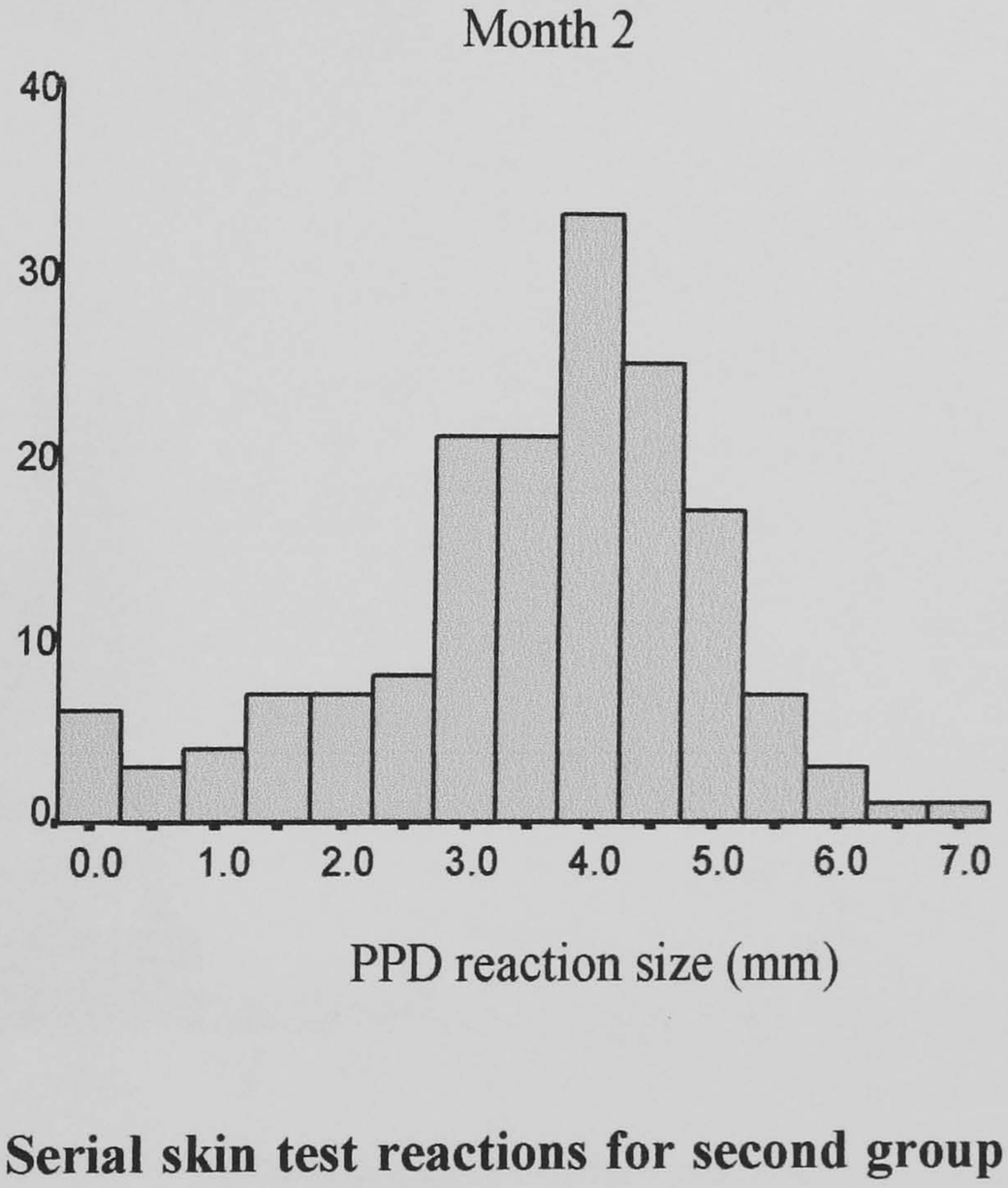


Figure 3-11: Serial skin test reactions for second group of animals over 2 month period in quarantine

PPD skin tests were performed on 160 animals resulting in mean induration diameter 3.4 mm for the first month shown in figure (a), and mean induration diameter 3.6 mm for 158 tests in the second month shown in figure (b).

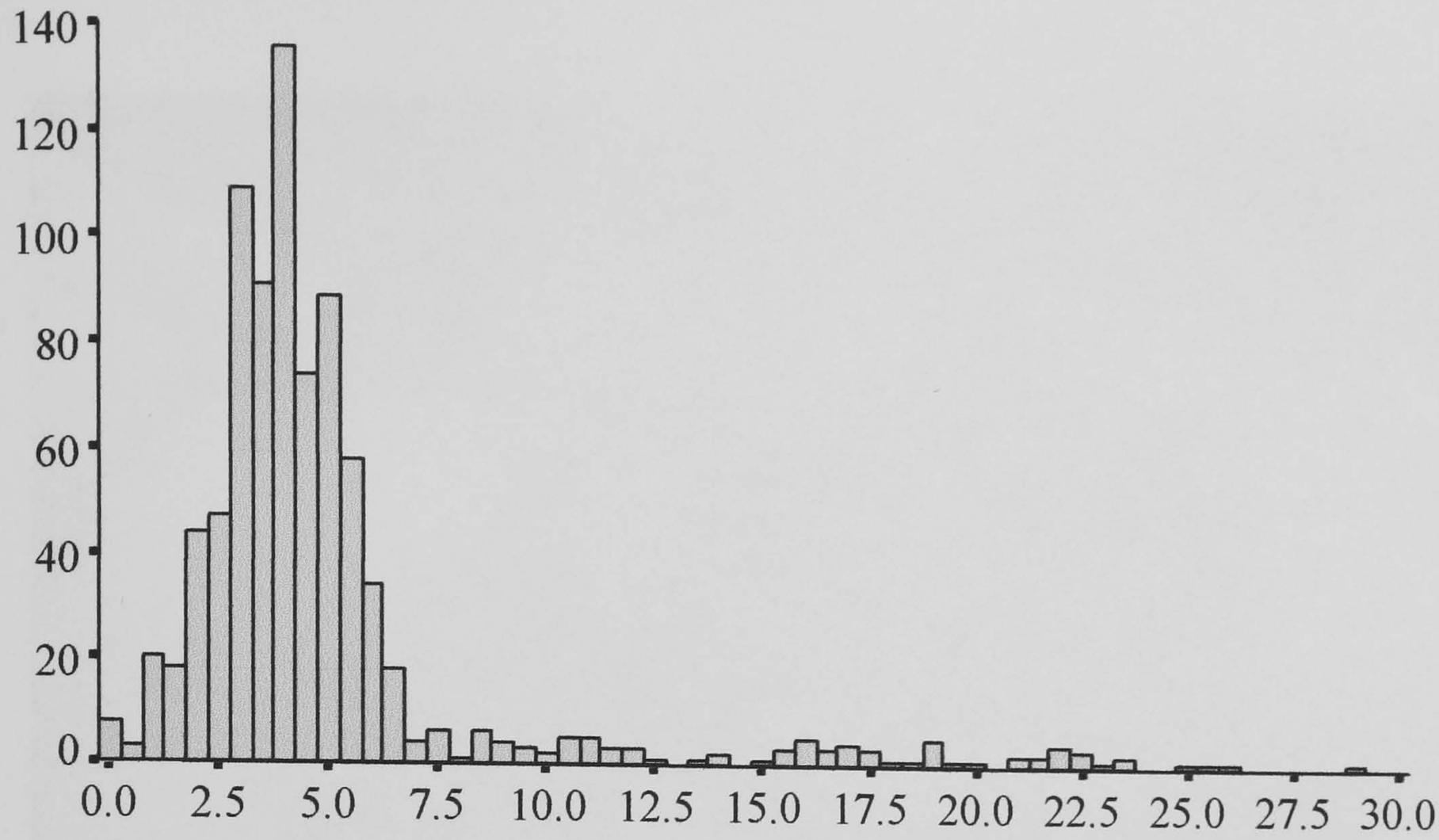


Figure 3-12: PPD skin test responses in male guinea pigs exposed to ward air

841 tests were performed on a total of 160 animals over a period of 16 months.

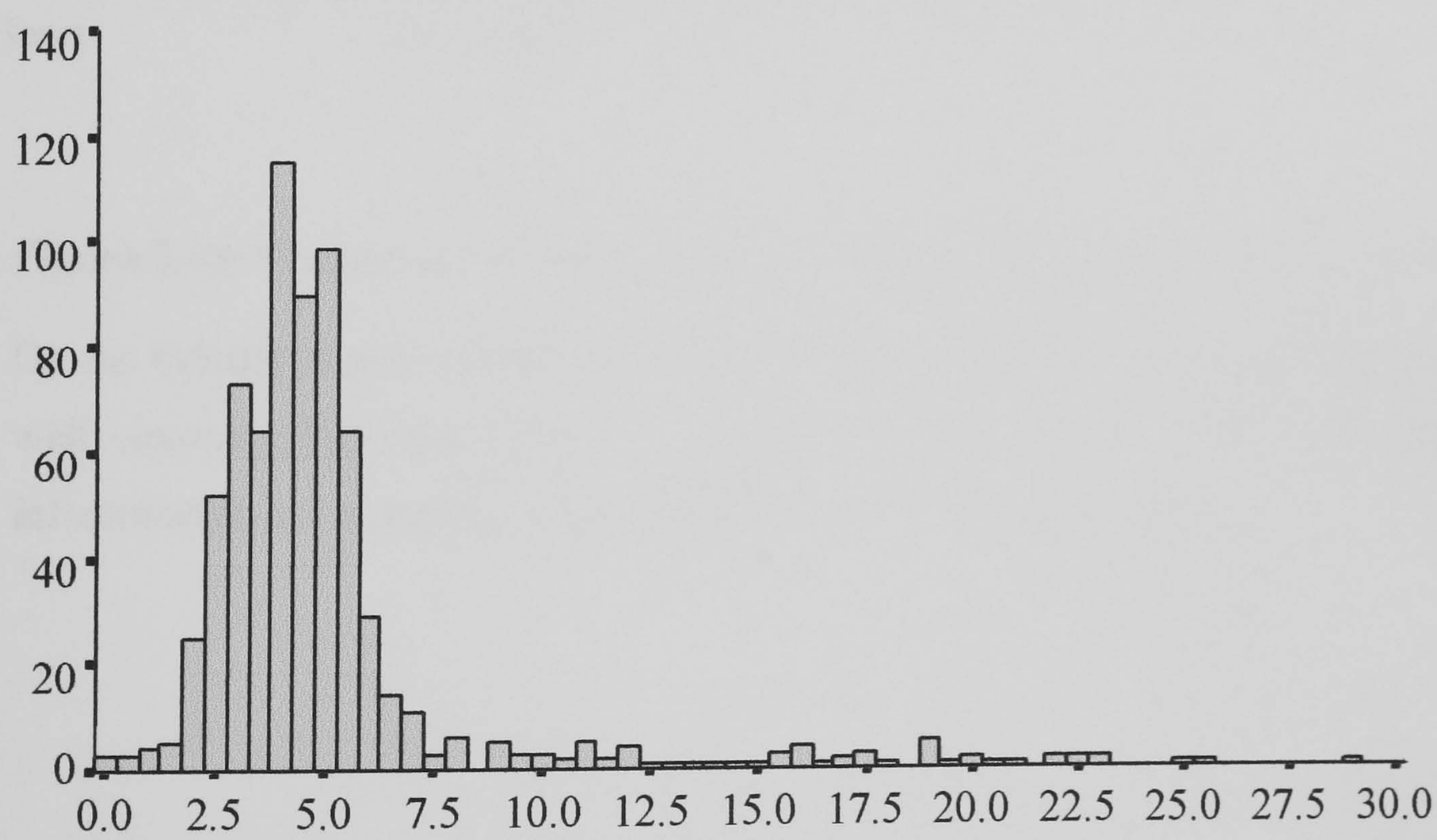


Figure 3-13: PPD skin test responses in female guinea pigs exposed to ward air

723 tests were performed on a total of 132 animals over a period of 16 months.

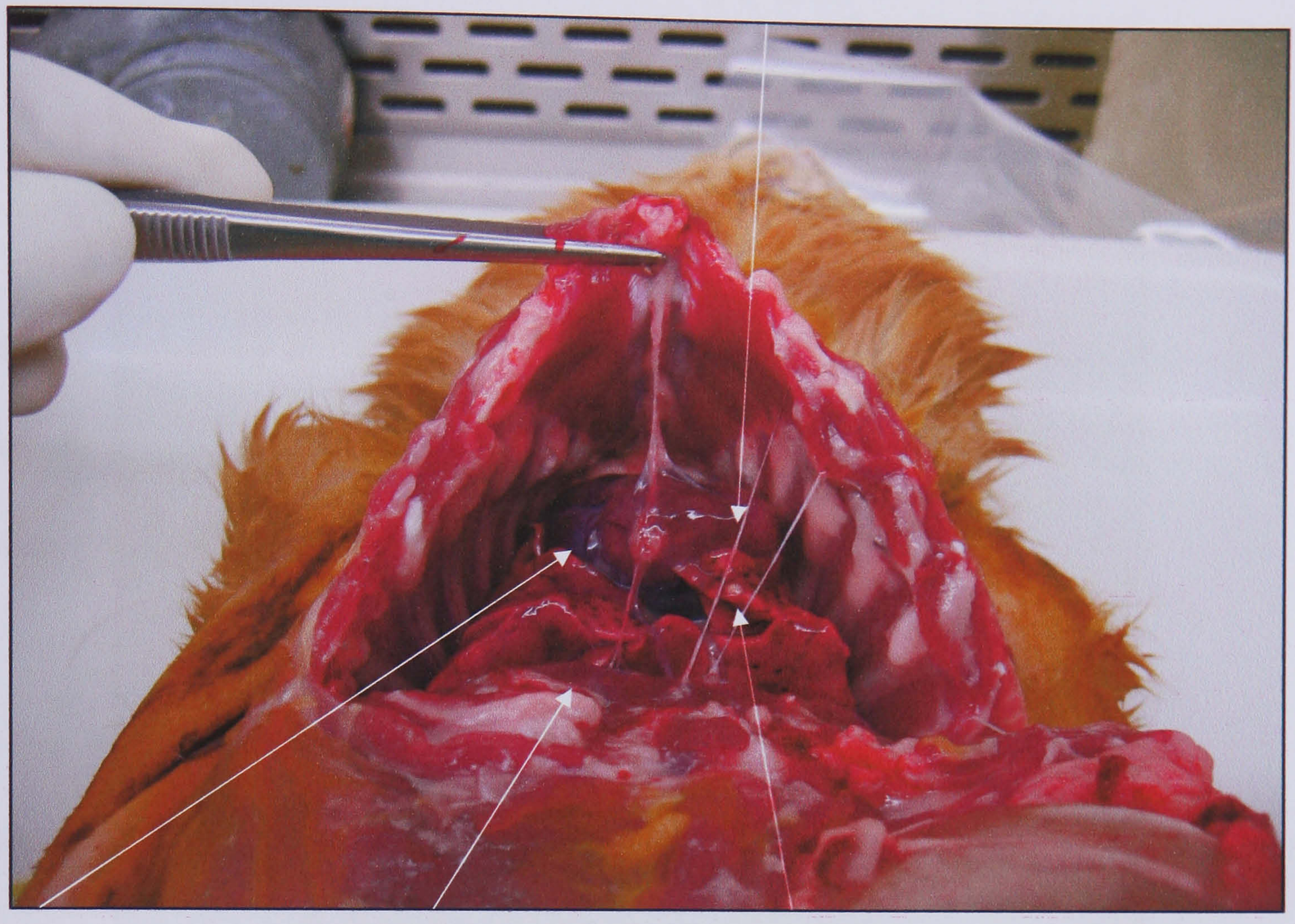


Figure 3-14: Guinea pig thoracic cavity showing a tuberculous primary focus

On the inferior surface of the left lateral lung lobe below the heart a 2 mm diameter well circumscribed white lesion is seen, the primary focus. There was associated inflammation of the pleura, and in addition a small pericardial effusion.

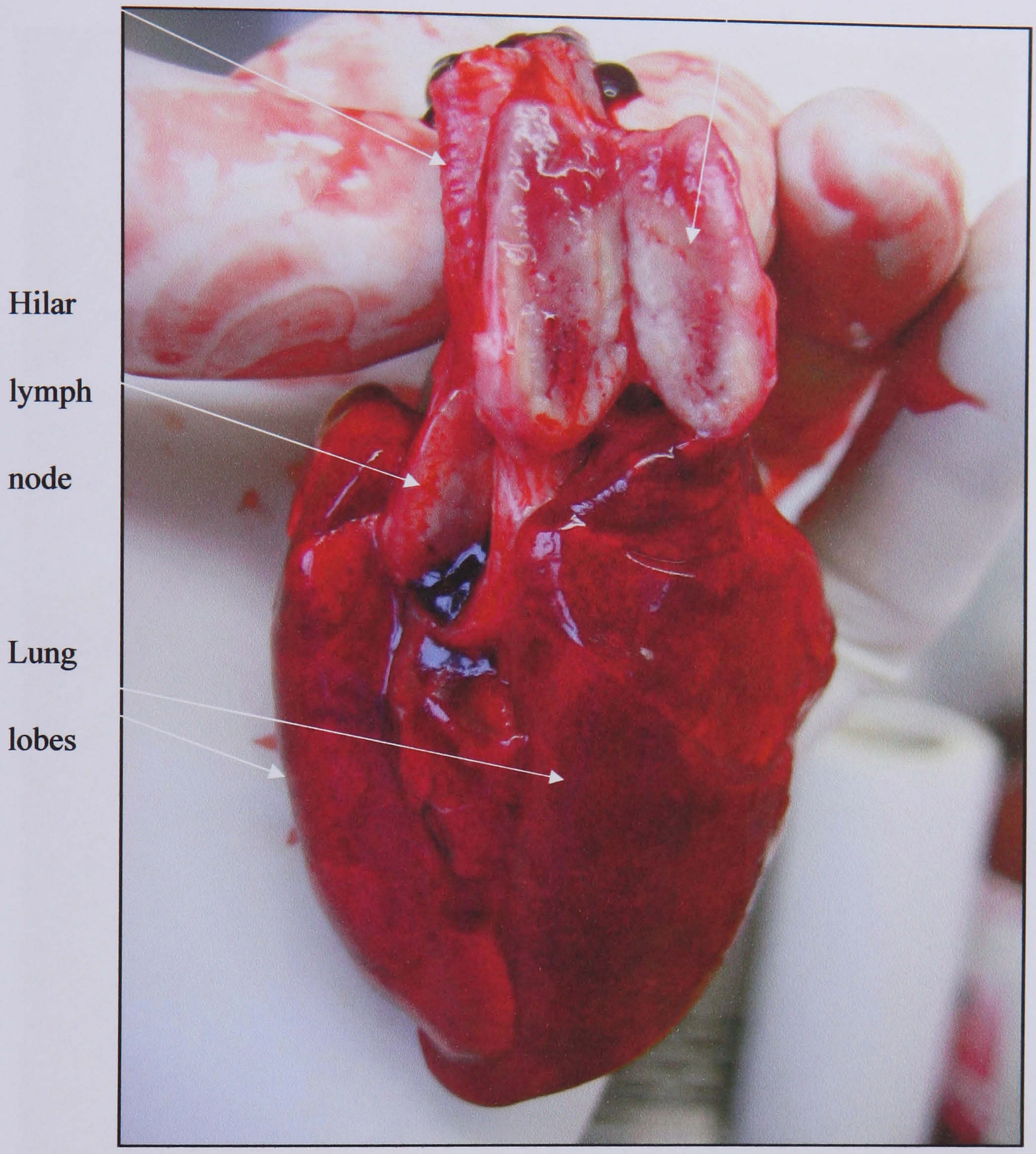


Figure 3-15: Tuberculous para-tracheal lymph node

A large lymph node 2 cm in length is seen on the right hand side of the trachea, and a smaller 1 cm lymph node is seen on the opposite side. Both have been sectioned to demonstrate the presence of hard white patches within these nodes, from which a soft creamy liquid emanated on applying light pressure, suggestive of tuberculous caseating necrosis.

Figure 3-16: TB involvement of spleen

The spleen of this PPD positive guinea pig is shown *in situ* at autopsy. Multiple pale foci were seen. On palpation these lesions were hard, and on section there was evidence of caseation (not shown).

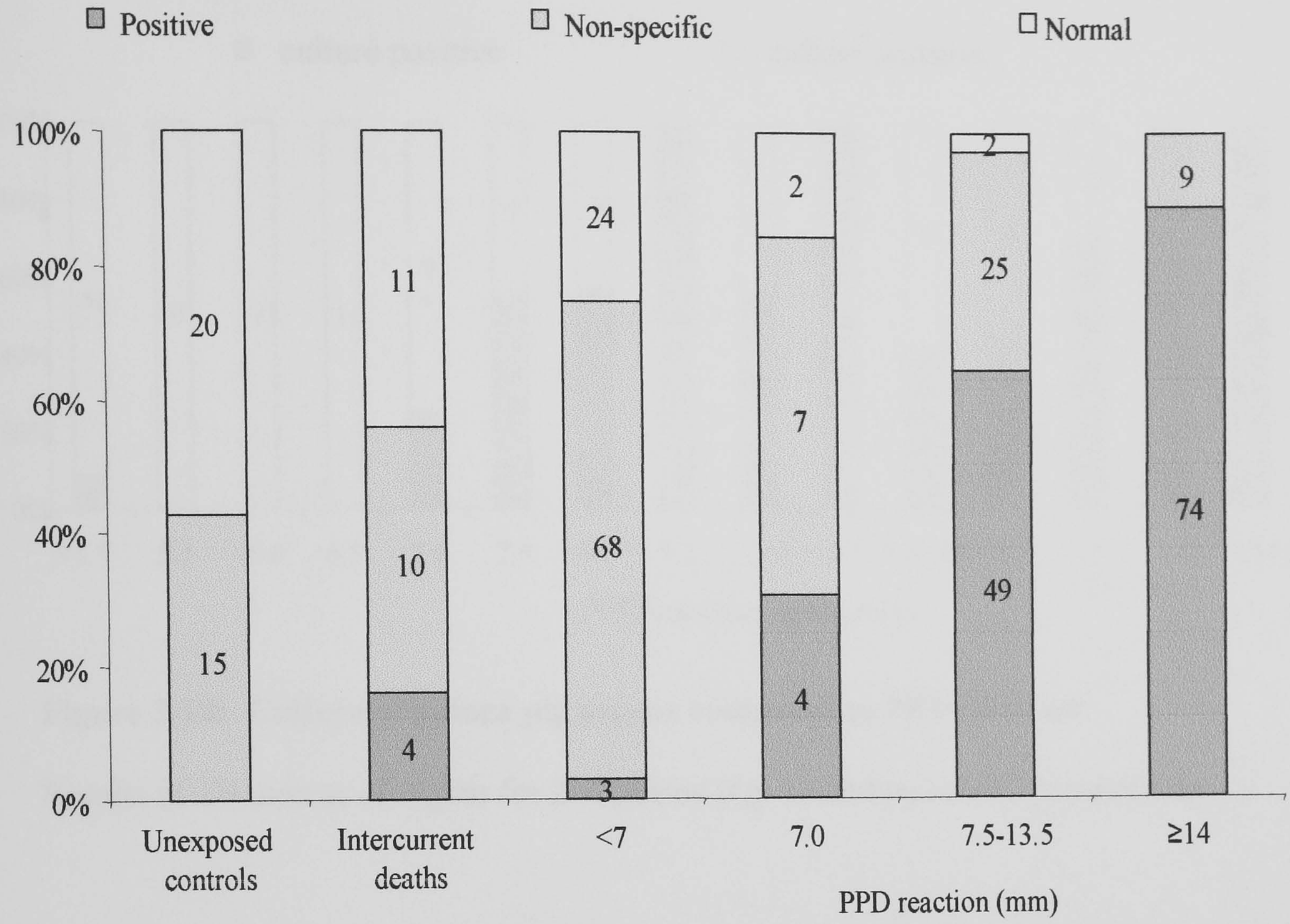


Figure 3-17: Autopsy results for sacrificed animals according to PPD skin test reaction; for intercurrent deaths; and for animals not exposed to ward air

The left hand two bars represent autopsy results (positive evidence of TB; abnormal with non-specific changes; or normal) for the unexposed negative control animals who breathed ambient roof air and for the exposed animals that died between skin tests ('intercurrent deaths'). The four right hand bars represent autopsy results for 267 animals exposed to ward air and sacrificed after a PPD skin test, grouped according to PPD skin test diameter.

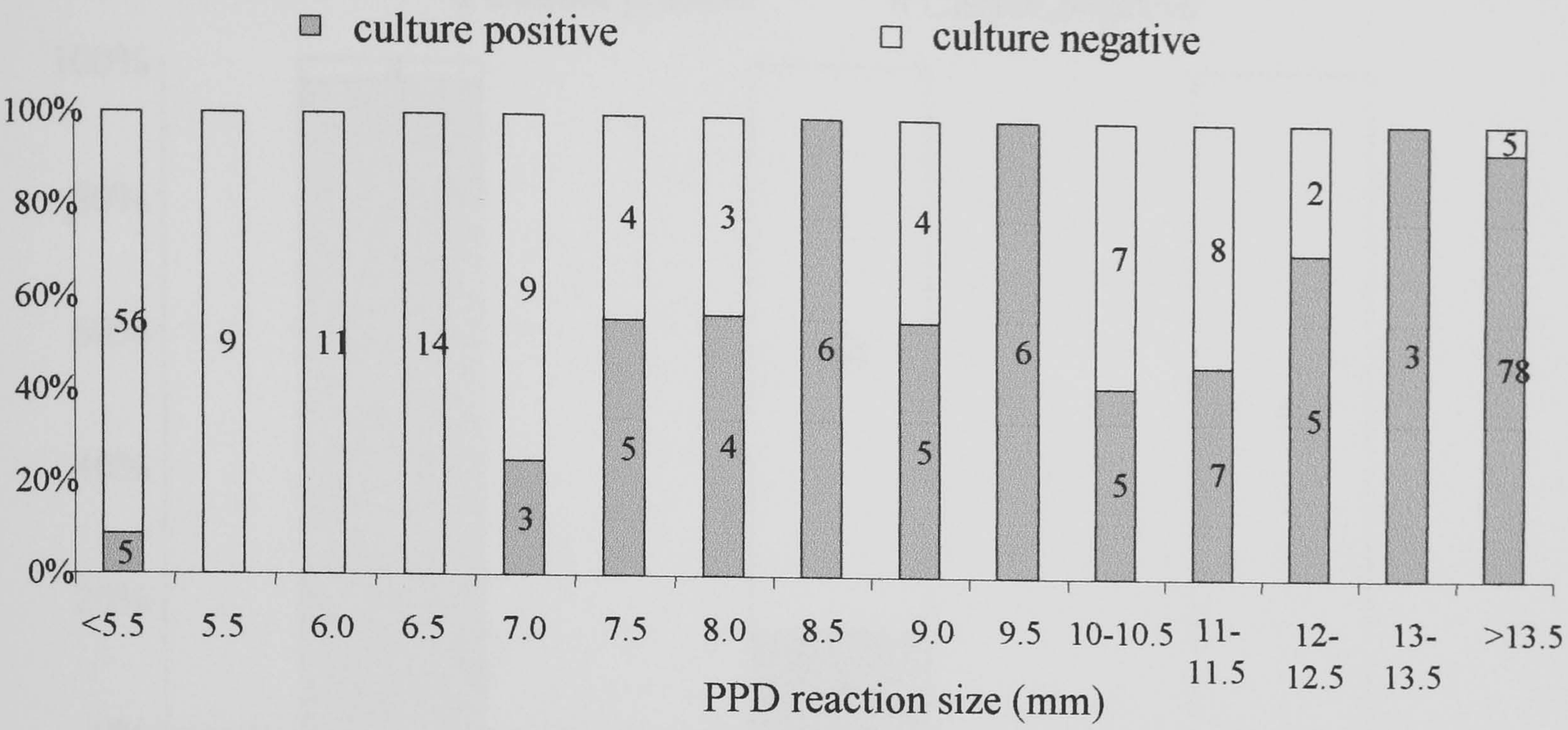


Figure 3-18: Culture of guinea pig organs compared to PPD skin test

Results of TB culture of organs for 264 guinea pigs according to PPD reaction size.

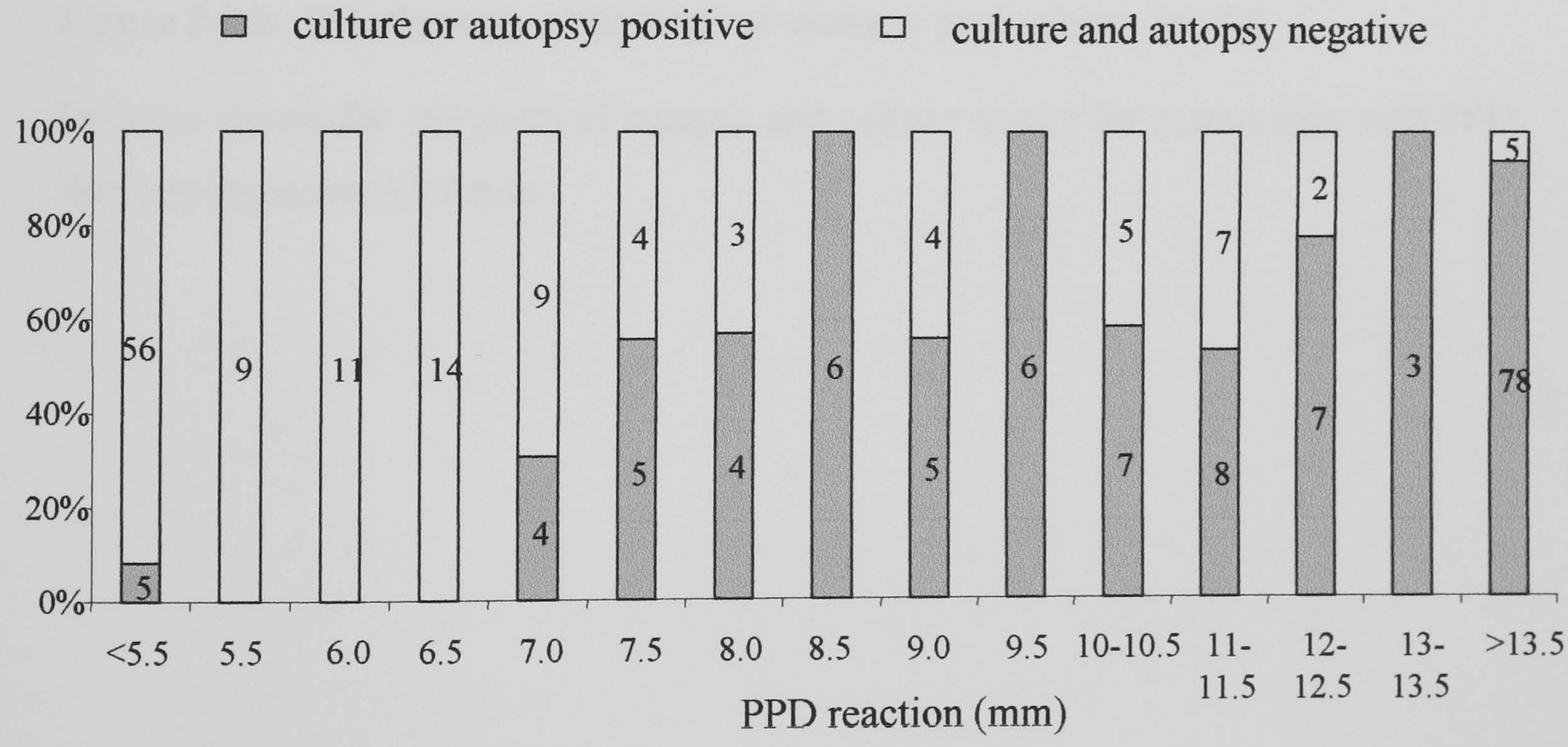


Figure 3-19: Culture and autopsy results combined compared to PPD skin test

Numbers of guinea pigs with positive organ culture or positive diagnostic autopsy for TB according to PPD reaction size, for a total of 267 animals.

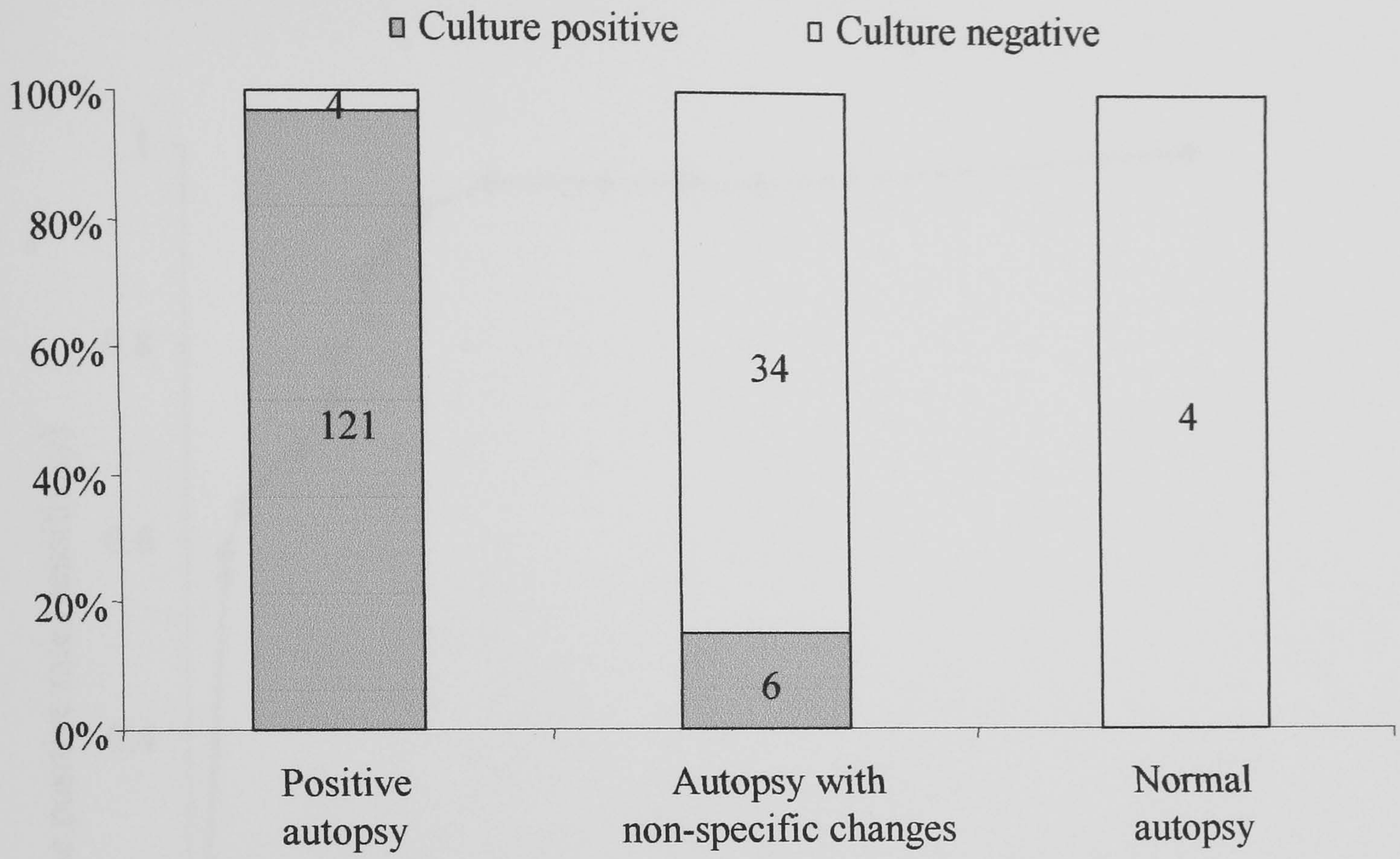


Figure 3-20: Relationship of findings at autopsy and culture for TB

Data are shown for 169 pairs of autopsy and culture results for guinea pigs with PPD skin test responses ≥7.0 mm.

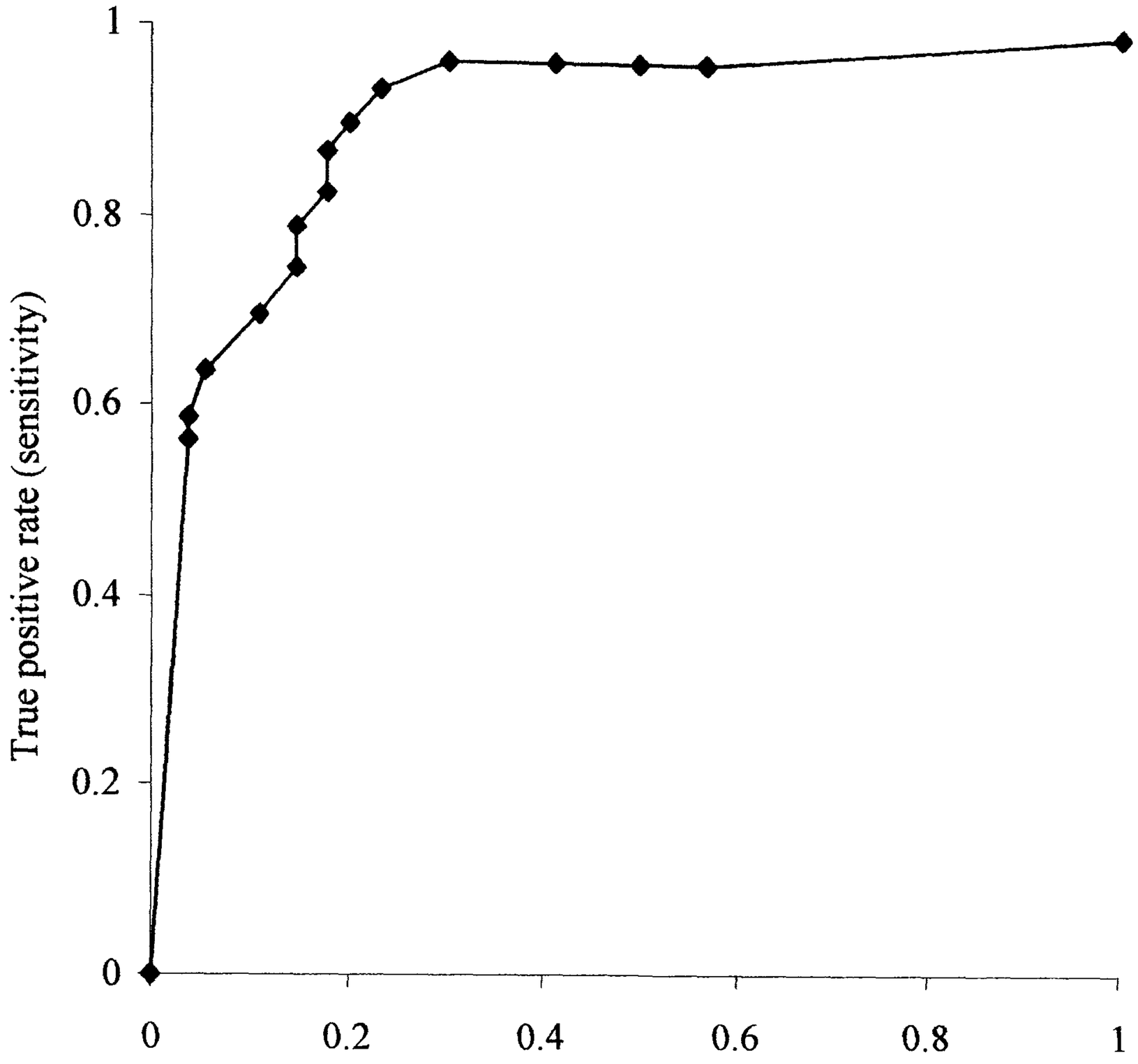


Figure 3-21: Receiver Operating Characteristic (ROC) Curve for 100 Unit PPD skin test in guinea pigs

ROC curve for PPD skin test in guinea pigs based on sensitivity and specificity calculated from combined autopsy and culture data for 267 animals. The area under the curve is 0.91, suggesting a test of 'excellent' accuracy.[166] Excluding the point at zero, from left to right points on the graph correspond to PPD cut-off points of ≥14, 13, 12, 11, 10, 9.5, 9, 8.5, 8, 7.5, 7, 6.5, 6 and 5.5 mm.

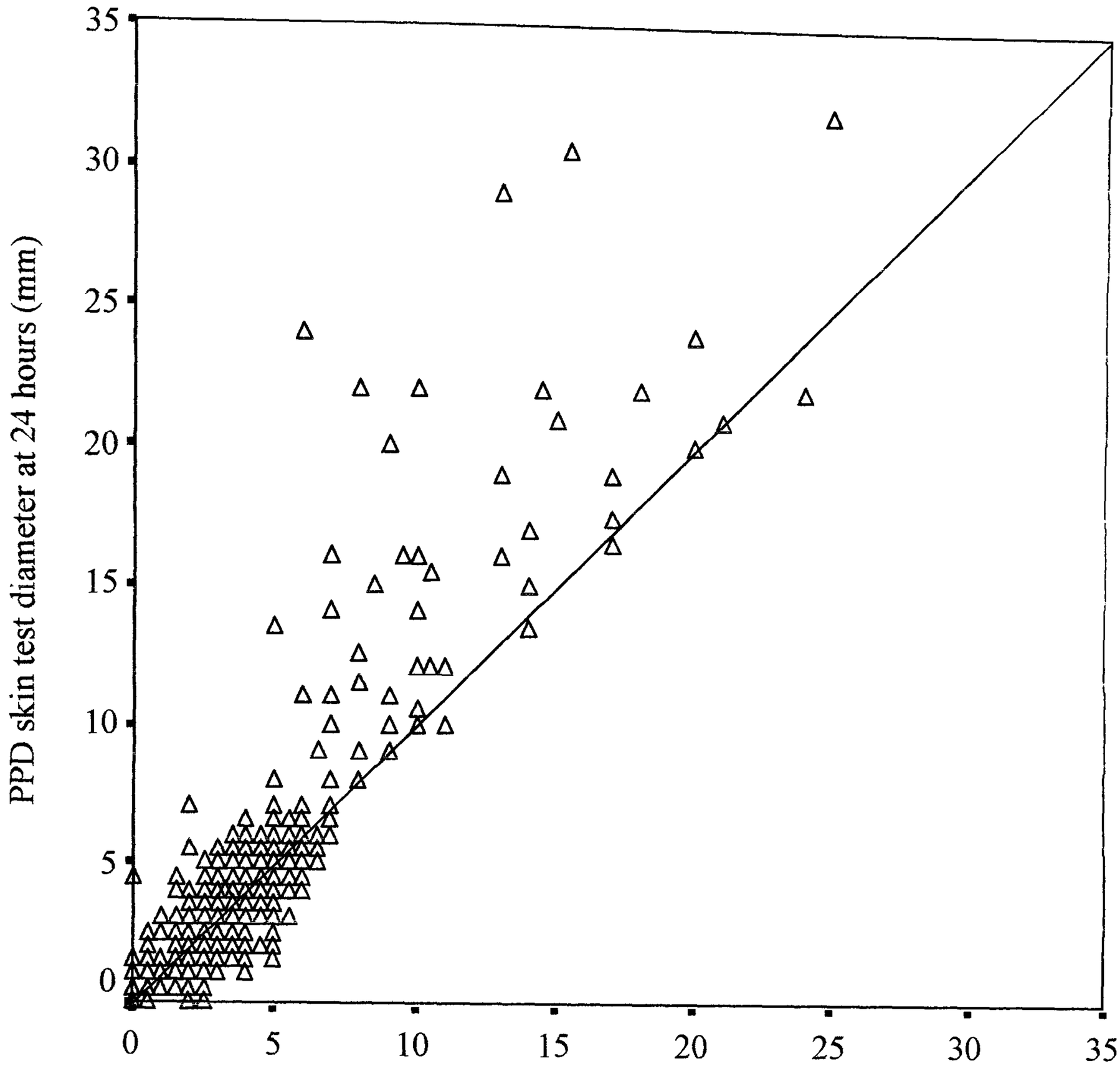


Figure 3-22: PPD skin test reactions at 24 *vs.* 48 hours

Readings were made at 24 and 48 hours for 917 PPD skin tests in a total of 604 guinea pigs in quarantine with no exposure to ward air and for 91 PPD skin tests in a total of 30 guinea pigs, either *M. bovis* positive controls or animals with presumed tuberculosis awaiting sacrifice. Readings identical at 24 and 48 hours would fall on the line drawn on the graph. The observed scatter of points to the left of this line indicated a group of readings that were greater at 24 compared with 48 hours.

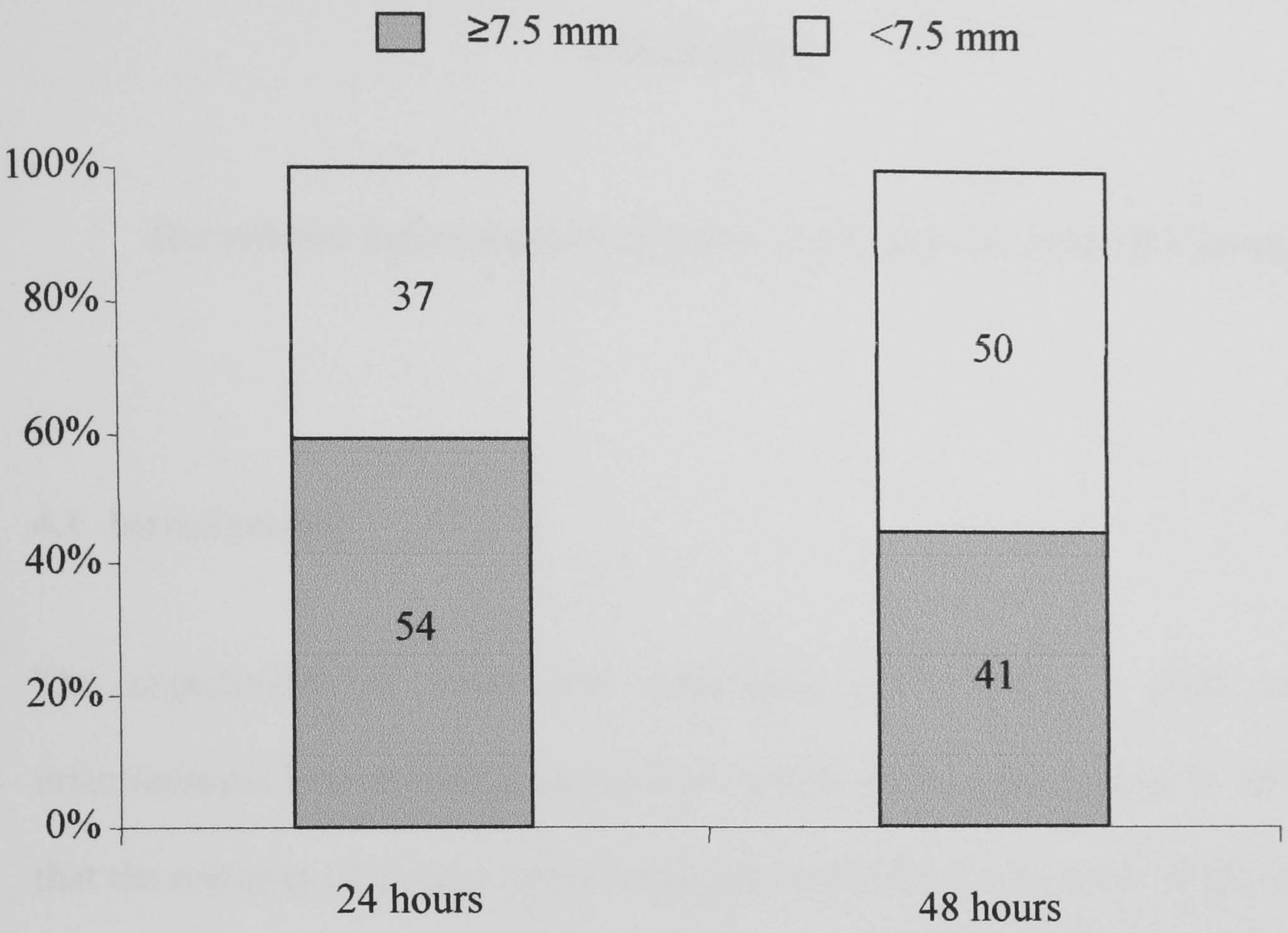


Figure 3-23: Difference in numbers of animals classed as positive at 24 *vs.* 48 hours for *M. bovis* positive controls or animals with presumed tuberculosis

The left hand bar represents 91 PPD skin test reactions read at 24 hours, of which 54 (59%) were ≥7.5 mm and therefore classed as positive. The right hand bar in comparison represents the same skin tests read at 48 hours, of which only 41 (45%) were ≥7.5 mm and would therefore be classed as positive (p=0.05).

CHAPTER 4

The relative infectiousness of tuberculosis patients with HIV co-infection

4.1 Introduction

The experiments of Riley and colleagues demonstrated a great variability in infectiousness between different patients infected with tuberculosis. It was also shown that the majority of disease transmission occurred from a minority of patients, and that the initiation of effective antimicrobial chemotherapy greatly reduced patient infectiousness. The infectiousness of patients was estimated from the time spent on the ward by the patient and the corresponding number of guinea pigs infected by that patient. This link between patient and guinea pig was determined by an identical pattern of drug resistance in both the guinea pig strain of *M. tuberculosis* and the patient strain of *M. tuberculosis*, and by the presence of that patient on the ward 3-7 weeks before the corresponding guinea pig became PPD positive and was removed from the exposure chamber.[75-77]

Since these original experiments on patient infectiousness, Riley and colleagues developed a model of airborne infection, which includes patient infectiousness ('q') as an important component, where 'q' denotes the number of infectious quanta produced per hour. This model is known as the Wells-Riley equation and has been described in previous chapters.[123] The Wells-Riley equation has subsequently been applied to

infectious tuberculosis cases reported in the literature. Riley calculated 'q' to be 1.3 on average for his patients over the four years of the entire study.[123] His most infectious case, a patient with laryngeal TB who infected 15 guinea pigs out of a total of 63, had a calculated value of q = 60.[123] Other cases from the literature include q = 13 for an untreated office worked who infected at least 40% of her colleagues over a four week period before diagnosis,[125] q = 250 for a bronchoscopy related outbreak[7] and q = 2280 for an outbreak on a surgical ward associated with jet irrigation of a tuberculous thigh abscess.[175,126] The first four of these values for q (1.3, 13, 60 and 250) have been used in Chapter 2 for modelling the effect of natural ventilation on airborne TB infection. These values for q can be put into the context of other diseases more infectious than tuberculosis; Riley calculated q = 5480 for measles based on a school outbreak, measles being a highly infectious disease transmitted by the airborne route.[123]

As discussed in Chapter 1, there are conflicting reports in the literature concerning the relative infectiousness of drug-sensitive *vs.* drug-resistant tuberculosis.[105-110] In addition there exists uncertainty as to the infectiousness of TB patients co-infected with HIV, owing in part to the greater prevalence of infiltrative disease compared with cavitation on the chest radiographs of such patients.[85-87]

In this chapter the relative infectiousness of TB patients co-infected with HIV and with a high prevalence of MDR-TB is investigated, building on Riley's original model using modern tools of molecular fingerprinting to determine which patients infected which guinea pigs, enabling the calculation of values of q for individual infectious patients.

4.2 Methods

4.2.1 Measurement of ward and animal house ventilation

Work was started on the ward renovation and construction of a negative-pressure respiratory isolation facility in the year 2000 and opened on 21 June 2001. The mechanical ventilation system was designed to deliver 6 air-changes/hour and negative pressure. The commissioning methodology has been described in Chapter 3 section 3.2.3, and the results are presented in this chapter.

4.2.2 Guinea pig air sampling

As described in the preceding chapter, two batches of guinea pigs were transferred to the exposure chamber after quarantine, 144 in May 2002 and 148 in November 2002. All exhaust ward air passed over the guinea pigs. Guinea pigs were PPD skin tested at monthly intervals and positive reactors (defined as $\geq$ 7.5 mm induration at 24 or 48 hours) were removed for sacrifice and culture of organs for TB. Cultures positive for *M. tuberculosis* were re-plated onto Middlebrook 7H-11 agar (Difco™, Beckton, Dickinson & Co., New Jersey, USA) and the resulting pure growth cryopreserved at -70 ^{0}C for DNA fingerprinting analysis (see section 4.2.4).

4.2.3 Patients

The ward functioned normally as part of the busy Servicio de Enfermedades Infecciosas y Tropicales (Infectious and Tropical Diseases Service) at Hospital Nacional Dos de Mayo in Lima. The study had no control over or effect upon patient admission to the eight beds under negative pressure where all exhaust air passed over

the guinea pigs. These eight beds were exclusively for HIV patients, who had either an existing diagnosis of TB or were suspected of having TB. Ward policy was to admit TB suspects to one of the beds in the first 4-bedded room until TB had been excluded, and to admit those with an existing diagnosis of TB to the second 4-bedded room, situated through self-closing double doors further down the corridor from the main entrance. All patients admitted to these eight beds were invited to participate in the study through informed written consent. Ethical approval for the study was obtained from the IRB of Asociación Benéfica PRISMA, Lima, Peru, Hospital Nacional Dos de Mayo, Lima, Peru, and Imperial College London, UK.

Questionnaires were administered to those patients providing informed consent to enter the study. These admission questionnaires included questions about past history of tuberculosis, duration of anti-tuberculous treatment and a report of the most recent chest radiograph if available. Shorter daily questionnaires were also completed, which recorded symptoms and signs of TB disease. These symptom questionnaires included the presence or absence of cough, cough frequency, whether the hand was used to cover the mouth during coughing for >50% of the time, whether a respirator or surgical mask was worn, sputum production, presence of fever, presence of haemoptysis, and the number of hours spent outside the room. 24 hour samples of sputum were collected. The volume of these samples was measured, and daily auramine sputum smears[164,176] and weekly sputum cultures were performed at the Laboratorio de Investigación y Desarrollo of Universidad Peruana Cayetano Heredia (UPCH). Sputum was decontaminated for 20 minutes using the NaOH-NALC method,[162] and cultured in Middlebrook 7H9 broth medium (Difco™) supplemented with oleic-acid, albumin, dextrose, and catalase (OADC; Remel, Lenaxa, Kansas,

USA) and an antimicrobial supplement PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin; Beckton, Dickinson & Co., New Jersey, USA) as previously described.[163] Cultures positive for *M. tuberculosis* were cryopreserved at -70 ^{0}C for DNA fingerprinting analysis (see section 4.2.4.) Drug sensitivity testing on *M. tuberculosis* isolates was performed as part of the routine laboratory sensitivity testing service using the Tetrazolium Microplate Assay (TEMA).[165]

Patients who did not consent to be part of the study had sputum collected as part of the ward policy of infection control. These samples were examined in the hospital microbiology laboratory for the presence of acid fast bacilli by Ziehl-Neelsen staining,[164] and cultured on Lowenstein Jensen solid media for *M. tuberculosis*.[164] Positive cultures were sent to the TB reference laboratory of the Instituto Nacional de Salud (INS) for drug sensitivity testing using the proportions method.[162] Basic details about these patients including diagnosis, site of tuberculosis disease and presence or absence of treatment were recorded for use in the study. The number of days spent on the ward by all patients was recorded, as was bed location.

4.2.4 DNA fingerprinting using spoligotyping

All available patient and guinea pig *M. tuberculosis* strains were typed using spoligotyping.[177] The steps detailed below were followed. All laboratory reagents came from Sigma Chemical Co., St Louis, Mo, USA, except where otherwise stated.

4.2.5 Extraction of DNA

100 µl of cryo-conserved *M. tuberculosis* was placed in a screw top tube and heated to 80 ^{0}C for 20 minutes to kill the bacteria. The tube was centrifuged at 10,000 rpm for 10 minutes and the supernatant discarded. 200 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8, 1% v/v Triton (Fisher Scientific)) and 20 µl of 10 mg/ml lysozyme (EC 3.2.1.17 from chicken egg white) were added and the mixture vortexed for 2-3 minutes to dissolve the pellet. The tube was subsequently incubated overnight at 37 ^{0}C in a shaker. 10 µl of 20 mg/ml proteinase K (Invitrogen Life Technologies, Carlsbad, CA, USA) were then added with 20 µl of 10% w/v sodium dodecyl sulphate (SDS; Bio-Rad Labs, Hercules, CA, USA), and the mixture incubated at 65 ^{0}C for 10 minutes, vortexing every 2 minutes. 50 µl of 5 M NaCl was added, followed by 50 µl of 10% w/v cetyltrimethylammonium bromide (CTAB; Aldrich Chemical Company, Milwaukee, Wisconsin, USA) previously heated to 65 ^{0}C. The resulting mixture was vortexed and incubated at 65 ^{0}C for 10 minutes. 350 µl of phenol-chloroform-isoamyl alcohol (25:24:1) was then added, and the mixture vortexed and centrifuged at 10,000 rpm for 5 minutes (chloroform: Merck, Darmstadt, Germany). The supernatant was transferred to another tube, and 350 µl of chloroform-isoamyl alcohol (24:1) was added, followed by vortexing and centrifugation at 10,000 rpm for 5 minutes. The supernatant was transferred to another tube and 2 volumes (600 µl) of cold absolute alcohol were added. The mixture was frozen at -70 ^{0}C for 15 minutes, and then at -20 ^{0}C overnight. Following centrifugation at 10,000 rpm for 8 minutes, the supernatant was removed and discarded. 0.5 ml of cold 70% alcohol was added to the pellet, and centrifuged at 10,000 rpm for 3 minutes. The resulting liquid was discarded, and the remaining pellet of DNA was dried, and then diluted in 50 µl of TE buffer.

4.2.6 DNA amplification

The following mixture was prepared for DNA amplification using the polymerase chain reaction (PCR): 1.5 mM $MgCl_2$; 50 mM KCl; 10 mM-Tris-HCl, Ph 8.3, Triton X-100 (1g/l; Fisher); 0.25 mM of each of dATP, dCTP, dGTP, dTTP (Invitrogen); 0.4 µM of primers DRa (GGTTTTGGGTCTGACGAC, 5' biotinylated) and DRb (CCGAGAGGGGACGGAAAC) (Isogen Lifescience B.V., IJsselstein, Netherlands); DNA (100-200 ng); 1 µl recombinant Taq polymerase (5 U/µl; Invitrogen). The following PCR cycle was used: 5 minutes at 95 ^{0}C; 25 cycles of 1 minute at 95 ^{0}C, 1 minute at 55 ^{0}C and 30 minutes at 72 ^{0}C; 5 minutes at 72 ^{0}C followed by cycle termination at 4 ^{0}C.

4.2.7 Hybridisation of PCR products on spoligotyping membrane

All buffers were prepared by dilution of stock concentrates with MiliQ water and pre-warmed in advance. 20x SSPE buffer was prepared using 175.3 g NaCl, 27.6 g $NaH_2PO_4H_20$, and 7.4 g EDTA in 1 litre of water; pH 7.4. PCR products were first heat-denatured by diluting 20 µl of PCR products in 150 µl of 2x SSPE/0.1%SDS buffer and heating to 100 ^{0}C for 10 minutes, followed by immediate cooling on ice. The spoligotype membrane (Kit IM9701; Isogen Lifescience) was prepared by repeated washing and rinsing. The first rinse was with 100 ml of 2x SSPE/0.1% SDS at 60 ^{0}C, followed by washing for 10 minutes in 200 ml 2x SSPE/0.1% SDS at 60 ^{0}C. This wash was repeated. The spoligotyping mini-blotter (Isogen Lifescience) was washed thoroughly with soap and a dedicated brush, and rinsed with 2x SSPE/0.1% SDS at 60 ^{0}C. The membrane was inserted into the mini-blotter such that the slots of the mini-blotter were perpendicular to the line pattern of the oligonucleotides

covalently bound to the membrane during manufacture. The blotter was screwed tight and residual fluid removed from the slots by careful aspiration. The slots were filled with 150 µl of diluted, denatured PCR product, taking care to avoid air bubbles and leakage from slot to slot and hence contamination of neighbouring lanes. Any unused lanes were filled with 2x SSPE/0.1% SDS at 60 ^{0}C. Hybridisation was performed by maintaining at the mini-blotter at 60 ^{0}C for 60 minutes on a horizontal surface. Following hybridisation, the samples were removed from the mini-blotter lanes by aspiration through the slots, and the membrane was removed and rinsed with 100 ml 2x SSPE/0.5% SDS at 60 ^{0}C. The membrane was washed for 10 minutes with 60 ml 2x SSPE/0.5% SDS at 60 ^{0}C. Finally the membrane was washed for 13 minutes in 100 ml 2x SSPE/0.5% SDS at 60 ^{0}C.

4.2.8 Detection of hybridisation on spoligotype membrane

Prior to incubation of the membrane with streptavdin-peroxidase, an initial rinse was performed in 100 ml of 2x SSPE/0.5% SDS at 42 ^{0}C, and this liquid was used to rinse a rolling bottle, and allowed to cool to prevent inactivation of the peroxidase in the next step. The membrane was placed in the rolling bottle, with printed side facing inwards. A 1:4000 dilution of streptavdin-peroxidase conjugate (500U/ml; Roche Diagnostics GmbH, Mannheim, Germany) was prepared by adding 18 µl of conjugate to 24 ml of 2x SSPE/0.5% SDS, and this was used to incubate the membrane in the rolling bottle at 42 ^{0}C for 45-60 minutes. The membrane was then rinsed in the same bottle with 100 ml of 2x SSPE/0.5% SDS at 42 ^{0}C, and then removed and washed two times for 10 minutes each in 200 ml of 2x SSPE/0.5% SDS at 42 ^{0}C.

The membrane was then rinsed in 100 ml of 2x SSPE, and then washed twice for 10 minutes at room temperature with 200 ml of 2x SSPE. Detection of the hybridisation of DNA was carried out using a chemiluminescent detection liquid. This was prepared by mixing 20 ml of each of the two detection liquids from the 'ECL detection kit' (Amersham Biosciences, Buckinghamshire, UK). The membrane was incubated for 3 minutes in this solution, ensuring that the entire membrane was submerged. Excess liquid was removed, and the membrane wrapped in cling film avoiding the formation of air or liquid bubbles. A sheet of X-ray film (Hyperfilm ECL; Amersham Biosciences) was exposed by placement adjacent to the membrane for 1 minute in a darkroom and developed according to the instructions of the manufacturer. To allow repeated use of the membrane, the hybridised DNA and detection products were stripped from the membrane. First, the membrane was rinsed in 100 ml of 0.95% SDS at 76 ^{0}C, and then it was washed twice for 30 minutes at 76 ^{0}C with 200 ml of 0.95% SDS. Membranes were sealed in a plastic box and stored at 4 ^{0}C until next use.

4.2.9 Determination of infectious patients

Infectious patients were determined by the following criteria: demonstration of an identical TB strain spoligotype pattern and drug sensitivity pattern to at least one infected guinea pig; and residence on the ward 3-9 weeks prior to the animal becoming PPD positive.

4.2.10 Calculation of patient infectiousness

The Wells-Riley equation $C = S\ (1\text{-}e^{-Iqpt/Q})$ was used to determine individual patient infectiousness (see Chapter 2 section 2.2.6 for a description of the terms included in

this equation).[123] The duration of exposure (t) was taken as the total number of days spent on the ward by the infectious patient. The number of susceptibles was the average number of guinea pigs resident in the animal facility exposed to ward air for the period that the infectious patient was on the ward. For guinea pig pulmonary ventilation a value of 8 cubic feet per day (0.227 m^3/day) was used.[76] For absolute ventilation, Q, the mean of measurements of air flow entering the animal house was used. Measurements were only considered for when there was a complete set of data available, i.e. measurements at all extraction vents leaving the ward, and measurements at all air injection vents entering the animal house. Allowance was made for the infiltration of outside air into the ductwork under negative pressure on its relatively long course from the ward up onto the roof of the two storey hospital building and into the animal house. The following re-arrangement of the Wells-Riley equation was used, with the introduction of the term 'r' to denote the leakage rate, where 'r' is a percentage of Q.[178,179] As the 'leakage' was in fact infiltration into ductwork under negative pressure, the value for 'r' was negative.

$$-\frac{\frac{Q(100-r)}{100}}{Ipt}\ln(1-\frac{C}{S})=q$$

4.3 Results

4.3.1 Measurements of ventilation in ward and animal house

A schematic representation of the ward including the ventilation system is shown in Figure 4.1. Four complete sets of ward and animal house ventilation measurements

were made using a balometer on two separate days. Mean total air leaving the ward was 851 cfm (SD 20 cfm). Mean total air entering the animal house was 973 cfm (SD 32 cfm). Thus air flow entering the animal house was greater than the total measured air extraction from the ward, and this reflected outside air infiltration into the ductwork which was under negative pressure relative to the atmosphere. Mean air infiltration was 12% (SD 3.5%) of the total entering the animal house. At the beginning of the study attempts were made to improve the sealing of ductwork, but this was hindered by the inaccessibility of some of the ductwork inside the false ceiling of the ward and behind wall installations. Negative pressure on the ward was measured to be between 0.001-0.002 inches of water. Direction of air flow into the ward rooms from the corridor was confirmed using smoke tubes. In smoke emitter testing to visualise air patterns in the animal house smoke entered the animal house, and dispersed rapidly and evenly to both sides of the central partition, without any clearly visible areas of air stagnation. Smoke testing in the animal facility also demonstrated minimal air infiltration around the door.

4.3.2 Total numbers of exposed guinea pigs and number of PPD positive animals at each monthly skin test

The number of exposed guinea pigs skin tested each month and the proportion that were PPD skin test positive (induration at 24 or 48 hours $\geq$7.5 mm diameter) is shown in Figure 4.2.

The difference in total numbers of guinea pigs between months reflects the removal from the exposure chamber of PPD positive reactors, PPD negative controls, and intercurrent deaths. Of note in this graph is the arrival of a new batch of 148 guinea

pigs in month seven to replenish animal numbers. To help define the cut-off between a positive and negative PPD skin test, 19 PPD negative animals with skin test responses 5–6.5 mm were sacrificed between month 2 & month 4, in addition to the randomly selected PPD negative controls. Also during this period there were 16 intercurrent deaths. Autopsies of these animals revealed extensive hepatic necrosis confirmed by histopathology. This was suspected to be due to possible pesticide use on the alfalfa used to feed the guinea pigs, so dry pellet feed was substituted. No further hepatic necrosis was observed.

There were no PPD positive animals observed in the skin tests of months 1, 13, and 16. There was just one PPD positive animal in month seven. The average number of PPD positive conversions each month was 10% (range 0-54%) of exposed animals tested. It can be seen in Figure 4.1 that a large number of PPD positive guinea pigs were detected in the month 10 skin test that was carried out on 26th March 2003. The research published by Riley and colleagues[75-77] implies that this was likely to reflect infection from a patient who was resident on the ward 3-7 weeks prior to the skin test, a hypothesis tested by DNA fingerprinting analysis presented in sections 4.3.7-4.3.9 and discussed in section 4.4.4.

4.3.3 Total patient numbers and patient diagnoses

Over the study period of 505 days (16.6 months) there were a total of 185 admissions by 161 patients. This resulted in total 2667 patient days in the two 4-bedded negative-pressure rooms where all air was exhausted to the guinea pigs. Thus average bed occupancy was 66%, and the monthly variations in bed occupancy by patient category are shown in Figure 4.3. For all patients, the median length of stay on the ward during

the study period was 11 days (inter-quartile range 6-21days), and the full range is shown in Figure 4.4.

There were 118 ward admissions by 97 patients with pulmonary TB, resulting in 1798 patient days (67% of total patient days); 33 ward admissions by 30 patients with extra-pulmonary TB, resulting in 609 patient days (23%); and 34 ward admissions by 34 TB suspects who subsequently had no laboratory evidence of TB resulting in 260 patient days (10%). Of the 68 admissions by patients who had extra-pulmonary disease or were TB suspects, 59 had sputum samples negative on smear microscopy and culture and nine were unable to produce a sputum sample. Of these nine patients, four were TB suspects who were subsequently moved from the negative-pressure facility to the general ward for patients with HIV, supporting the assumption that they were unlikely to have had active TB. Of the 97 total pulmonary TB patients, 13 had multiple admissions. Eight patients had two admissions, three patients had three admissions, and two patients had four admissions.

All patients were HIV positive. Of the patients with pulmonary TB, some were admitted for TB diagnosis followed by initiation of treatment, others were admitted owing to the adverse effects of anti-tuberculous chemotherapy, others were admitted for having abandoned treatment altogether and the remainder were admitted for other complications of their TB or HIV disease. The study had no influence on patient admission to the ward or duration of hospital stay. Patients consented to participate in the study in 74 (40%) of a total of 185 ward admissions.

4.3.4 Pulmonary TB patients: sputum smear status and TB drug sensitivity

Pulmonary TB cases were defined as those with evidence of acid-fast bacilli on sputum smear microscopy or those positive for *M. tuberculosis* on sputum culture. Sputum smear status (auramine staining) and culture results were available from the laboratory at UPCH for study participants, or through the hospital microbiology department (Ziehl-Neelsen in place of auramine staining for acid-fast bacilli) and INS laboratory for non-participants. Some patients arrived on the ward with an existing diagnosis of pulmonary TB made elsewhere, based on a sputum smear test at a health post or another hospital, and were already on treatment for TB. TB cultures on these patients were usually negative, and these patients were classed as 'presumed drug-sensitive' or 'presumed drug-resistant', according to their clinical histories and treatment regimens and responses.

The TB drug sensitivity and sputum status of 95 of the 97 pulmonary TB patients are shown in Figure 4.5. Two patients are not included: one drug-sensitive culture positive patient (accounting for 6 patient days) who had an unavailable smear result; and one smear negative, culture positive patient who did not have a TB sensitivity result (accounting for 8 patient days). Of the 32 patients with confirmed drug-resistant disease, 22 (69%) had multidrug-resistant strains, 8 (25%) had isoniazid mono-resistant strains and 2 (6%) had rifampicin mono-resistant strains. In the text and tables and figures, 'drug-resistant' refers to both multidrug-resistant and mono-resistant cases unless otherwise specified.

Although there were a greater number of drug-sensitive cases resulting in more patient days than drug-resistant cases (58 cases; 956 patient days *vs.* 46 cases; 828 patient

days respectively), drug-resistant cases were responsible for a greater number of sputum smear positive, culture positive patient days (403 *vs.* 215 days).

4.3.5 Pulmonary TB patients: treatment status

Table 4.1 shows the treatment status of ward admissions with pulmonary TB disease. There were 69 admissions for cases with drug-sensitive TB, for which treatment is categorised into 'established', of greater than two weeks duration; 'recent', commenced in the last two weeks; and 'new', commenced during the ward admission. A further category of 'none' denotes those patients who had abandoned treatment, or for whom it had been withdrawn due to adverse effects. It can be seen that new drug-sensitive cases accounted for 323 days on the ward (18% of total pulmonary TB patient days) of which 77 (4% of total pulmonary TB patient days) were before treatment was started, whilst the diagnosis of TB was being made.

There were 50 admissions for cases with drug-resistant TB, for which treatment is further sub-divided into 'correct' or 'sub-optimal'. 'Correct' signifies an antibiotic regimen suitable for the TB strain drug resistance pattern, whilst 'sub-optimal' signifies treatment with a regimen less likely to result in cure. This included treatment with standard first line therapy designed for drug-sensitive disease (two months of isoniazid, rifampicin, ethambutol and pyrazinamide followed by four months of isoniazid and rifampicin)[4] administered to drug-resistant cases whilst indirect drug sensitivity results were awaited, a process commonly taking up to 2 or 3 months. The sub-optimal treatment group also included those patients with single isoniazid or rifampicin resistant strains or multidrug-resistant strains placed on 'Esquema 2' of the Peruvian National Control Programme for TB, a regimen containing standard first-line

drugs with the addition of streptomycin.[4] Patients who failed standard first line therapy were treated with 'Esquema 2' until March 2001 when policy was changed and such patients were transferred directly to a standardised 18 month regimen containing the second line drugs kanamycin, ciprofloxacin and ethionamide, given with pyrazinamide and ethambutol.[4] However, within the programme there were difficulties in maintaining regular supplies of second line drugs.

It may be seen from Table 4.1 that new drug-resistant TB cases were responsible for fewer patient days than new drug-sensitive TB admissions (214 (12%) *vs.* 323 (18%)). However, 274 (56%) of 489 drug-resistant TB patient days with recent or established treatment were by patients on sub-optimal treatment.

4.3.6 Guinea pig TB drug resistance patterns

Table 4.2 shows the drug resistance patterns available for culture positive guinea pigs using the tetrazolium microplate assay method.[165] The spoligotype pattern number (see section 4.3.7) is also shown. It can be seen from the table that of the 108 culture positive guinea pigs with identical spoligotype pattern (#7) from the large outbreak in months 10-12, drug sensitivity results were available in 52. Of the four guinea pigs in month 14 with spoligotype pattern #7, no drug sensitivity results were available owing to bacterial contamination of the assay, and these animals were denoted as having presumed drug-resistant TB because they shared the same spoligotype pattern as an MDR strain. This pattern was also observed in a patient on the ward in month 13, whose drug sensitivity pattern was MDR.

4.3.7 Guinea pig TB spoligotype patterns

Of 135 guinea pigs culture positive for TB, spoligotype results were available for 125. Eight different spoligotype patterns were observed. Figure 4.6 shows a typical spoligotype film. Each of the 43 rows corresponds to one sample. Figure 4.7 shows the temporal distribution of these guinea pig TB spoligotype patterns according to the monthly skin test at which the guinea pig was diagnosed as PPD positive or was sacrificed as a control animal and was subsequently found to be a false negative. If the strain arose from an intercurrent death, it is included in the subsequent skin test group. It can be seen that there was a large monoclonal outbreak in month 10 of a strain with spoligotype pattern #7, which continued to be seen in the skin tests of month 11 and month 12. The same spoligotype pattern reappeared in months 14 and 15, but was not observed in month 13.

In 10 guinea pigs, spoligotyping was performed on positive TB cultures from all three separately cultured tissues: lymph nodes, lung and spleen. In all 10 animals, spoligotype patterns were identical in all three tissues. In nine animals where a combined specimen of lymph node and lung was cultured, with spleen cultured separately, spoligotyping was performed on both resulting strains, and again spoligotype patterns were identical in all tissues. No guinea pigs had discordant spoligotype results, i.e. different patterns in *M. tuberculosis* strains isolated from different tissues. In nine animals with more than one pulmonary primary focus of TB, two foci were dissected individually from different lung lobes with separate sets of sterile instruments and cultured separately. In all nine cases, both of the primary foci yielded the same spoligotype pattern.

4.3.8 Patient TB spoligotype patterns

Spoligotype results were available for 49 (42%) of the 118 pulmonary TB admissions, corresponding to 39 (40%) of the 97 different pulmonary TB patients. Some of the patients who did not enter the study had a spoligotype pattern available through sputum specimens already in our research laboratory as part of other studies being conducted on out-patients at Dos de Mayo Hospital. Patient TB strain spoligotype results were only considered for sputum samples obtained during or within 2 months of the hospital admission in which TB transmission to the guinea pigs could have occurred. The remaining admissions were mainly by patients with culture negative sputum, or patients who were unable to produce a sputum specimen. 16 patients, of whom four were sputum smear positive, had culture positive sputum, but no spoligotyping result. This was either due to laboratory contamination during processing or lack of availability of the strain because the reference laboratory did not routinely store patient TB strains. For the 49 patient admissions with spoligotype results available, 23 different spoligotype patterns were observed.

4.3.9 Determination of infectious patients

Of the 125 guinea pigs with spoligotype results, it was possible to match 122 (98%) with a ward patient with a TB strain with an identical spoligotype pattern who had been on the ward in the 3-9 weeks prior to the guinea pig's diagnosis. This resulted in the identification of 10 different infectious patients, with a total of seven different spoligotype patterns. Of these seven spoligotype patterns related to 10 clusters of infected guinea pigs, one pattern was seen in only one patient, and the remainder were seen in more than one patient during the whole study period. However, in the 3-9

weeks prior to the infection of each guinea pig cluster, only one patient was identified on the ward with that particular pattern.

There were at least two further infectious patients. One infected two guinea pigs that had a unique spoligotype pattern not seen amongst the 23 patterns available for patients. These two guinea pigs had MDR-TB, and during the 3-9 week period prior to their infection there were two sputum smear-negative culture positive MDR-TB patients and one sputum smear-positive culture positive MDR-TB patient on the ward for whom spoligotype results were unavailable. Any of these patients could have been the source of infection in these guinea pigs. The second unidentified infectious patient caused a guinea pig to become PPD positive in month 5, with an MDR strain with spoligotype pattern #1 (coloured red in Figure 4.7) that had also appeared in month 3. The three MDR-TB patients mentioned above could also include this second unknown infectious patient, as the animal infections occurred at approximately the same time.

4.3.10 Characteristics of infectious patients

Of the 97 pulmonary TB patients on the ward during the study, 10 were shown to have caused infection in the guinea pigs, with a further two infectious patients remaining unidentified. The characteristics of the 10 identified infectious patients and two unidentified infectious patients are shown in Table 4.3. Nine of the infectious patients had drug-resistant disease and three had drug-sensitive disease. Of the 10 identified infectious patients, all were sputum smear positive except for one patient with drug-sensitive TB whose sputum was smear negative but culture positive. Four had new diagnoses of TB, and thus spent time on the ward untreated before diagnosis and initiation of treatment. Two had treatment suspended as a result of adverse effects, and

treatment was re-initiated in only one of these cases, patient #12, who had MDR-TB. Four patients had recently been started on treatment for drug-resistant disease with sub-optimal treatment regimens, and one patient was on established sub-optimal treatment with first-line anti-tuberculous drugs[4] for MDR-TB disease. Table 4.3 also includes a column for patient infectiousness, q, which is discussed in the following section.

Of the 10 identified infectious patients, eight were study participants and chest radiograph reports were available for six. All had evidence of parenchymal infiltration or pneumonia, and none had evidence of cavitation. The patient responsible for the large outbreak in months 10-12 had a highly productive cough, producing 10-90 ml of sputum daily, for the first two weeks of a 32 day ward admission, the first 11 days of which were without treatment. The patient responsible for the second outbreak with a strain with this same spoligotype pattern, seen in months 14 and 15, also had a highly productive cough, producing 10-50 ml of sputum daily throughout a 26 day admission. This compares with a median maximum daily sputum volume of 24 ml (inter-quartile range 2-38 ml) amongst non-infectious patients (n=25), and 20 ml (inter-quartile range 6-44 ml) amongst infectious patients (n=8) for whom data is available (p=0.7; Mann-Whitney U test).

Univariate logistic regression analysis was performed for putative determinants of infectiousness for pulmonary TB patient admissions for which a spoligotype pattern was available, as seen in Table 4.4. Analysis was performed on admissions rather than individual patients, as patients with multiple admissions changed sputum smear status or treatment status between different admissions, and one patient with drug-sensitive TB acquired MDR-TB. Admissions were only included if a spoligotype result was

available for a sputum sample collected during that admission itself, or within two months of the admission, assuming the same TB illness episode. The analysis therefore included 49 ward admissions (corresponding to 39 different patients), of which 10 were classed as infectious, resulting in TB transmission to the guinea pigs, and 39 were classed as non-infectious. Characteristics of patient ward admissions that were significantly associated with TB transmission to the guinea pigs included sputum smear positivity ($p=0.05$), MDR-TB *vs.* drug-sensitive TB ($p=0.01$) and sub-optimal treatment ($p=0.02$). For this analysis, the sub-optimal treatment category included four patients who had abandoned treatment or for whom treatment had been stopped owing to adverse effects. If these four patients were excluded (hence $n=9$ and $n=36$ for infectious and non-infectious admissions respectively), sub-optimal treatment remained significantly associated with TB transmission to the guinea pigs in univariate analysis ($p=0.02$). Multiple regression analyses were not performed because of borderline sample size and significant co-linearity between the independent variables sputum smear positivity, MDR-TB *vs.* drug-sensitive TB, and sub-optimal treatment that were significantly associated with TB transmission to the guinea pigs.

A second univariate logistic regression analysis was performed on a subset of 33 patient admissions for which details of daily symptoms were available since the patients had consented to enter the study. These putative determinants of infectiousness included cough frequency, whether the hand was used to cover the mouth whilst coughing, haemoptysis and fever. None of these factors approached significance in univariate regression (all $p>0.6$) which may reflect the small sample size. Chest X-ray findings were not included in analyses because reports were only available for 17 patient admissions.

4.3.11 Relative infectiousness of patients: infectious quanta per hour

Table 4.3 also shows a value of q, the number of infectious quanta produced per hour, calculated for each of the 12 infectious patients. For the absolute ventilation in the Wells-Riley equation (Q), the mean (+/- 1 SD) of the total air injection into the animal house was used, i.e. 973 cfm (+/- 1 SD = 32 cfm). Each value for total ventilation Q (m^3/h) was adjusted for the percentage infiltration of outside air as described in the methods section. Mean infiltration of outside air between the ward and the animal house was 12% (SD 3.5%). However, the total air leaving the ward also included exhaust air from a small 978 cubic feet day clinic room adjacent to the two rooms for TB patients used in the study. This clinic room was used in the mornings to deliver intravenous therapies to non-TB patients and was empty in the afternoons. The mean air extraction from this room was 120 cfm (SD 2 cfm). This room had its own supply of fresh air, and because the exhaust air from this room was unlikely to be contaminated with TB, this 120 cfm was also considered as 'infiltration' of outside air. The total percentage infiltration was therefore increased to 25% (SD 3%).

The patient responsible for the outbreak of months 10-12 produced on average 281 infectious quanta per hour (range 272-291 when using in calculations the mean value for Q (m^3/h) plus or minus one standard deviation) if the 108 animal infections with matching spoligotype patterns are used as the number of new cases. This rises to q=357 (346-369) if the 10 animals culture positive in this period but without a spoligotype result and the five autopsy positive guinea pigs without culture and spoligotyping are included as assumed to be part of the same monoclonal outbreak.

The value of infectiousness calculated for all patients over the 16 month study period was 7.9 infectious quanta per hour, using as new cases the total 138 animals with autopsy or culture confirmed TB detected over the entire air sampling period. If the PPD positive but autopsy and/or culture negative animals are also included as new cases (168 animals in total) the value of q increases to 9.8. The median value of q for the 10 identified infectious patients was 15 (inter-quartile range 4-54).

4.3.12 Spatial distribution of guinea pig TB infection in the animal house: evidence for airborne spread of disease

Figure 4.8 is a schematic representation of the animal facility. Of note is the central partition, which divided the facility in half, such that infected air from the ward passed approximately equally down each side. This reduced the possibility of horizontal airborne spread of TB between guinea pigs between right and left sides. On one side of the partition, a guinea pig 'downwind' of an infected guinea pig in one cage could theoretically be infected by an animal 'upwind'. However, for this to be the case, the following conditions would need to be met: the 'index' guinea pig would have an identical spoligotype pattern to a patient resident on the ward 3-9 weeks before the index guinea pig became PPD positive; and the secondary guinea pig case would become PPD positive at least 3 weeks after the index case became infectious. Lurie's experiments showed guinea pigs infected by the airborne route may take many months to become infectious.[173,174]

Figure 4.9 shows five pairs of guinea pigs with identical TB spoligotype patterns. For the first three pairs, infection occurred either side of the central partition, and thus horizontal transmission between guinea pigs was highly unlikely. The fourth and fifth

pairs of infections occurred on the same side of the partition, but arose at similar time points, again making the likelihood of horizontal spread remote for the reasons explained above. Figure 4.10 shows the spatial distribution of infection observed in study months 10, 11 and 12. The spatial distribution of infection was random, with infection occurring on both sides of the central partition and in both upper and low level cages, consistent with airborne transmission from an infectious source on the ward.

4.4 Discussion

This chapter has investigated the relative infectiousness of pulmonary TB patients co-infected with HIV, with a high prevalence of multidrug-resistant TB. Over 16 months a heterogeneous mix of HIV patients with pulmonary or extra-pulmonary TB at different stages of their disease, in addition to HIV patients with suspected TB, were admitted to the ward. This was an operational ward in an HIV-TB service and the study had no influence over the decision to admit patients or the duration of ward admissions. The infectiousness of these patients calculated for the whole study period was between 7.9 and 9.8 infectious quanta per hour, more than six times the average infectiousness calculated for the patients studied by Riley over the four years of his study. This value calculated for the whole study period masks a wide variability in infectiousness of patients however, with only 12 of 97 pulmonary TB patients likely to have infected the guinea pigs, with one highly infectious MDR-TB case having a calculated value of $q = 281$. The strengths and implications of these findings are discussed below.

4.4.1 Measurements of ventilation

All calculations of patient infectiousness are dependent on reliable measurements of ward and animal house ventilation. Only four complete sets of ward and animal house ventilation measurements conducted on two different days were made owing to the availability of the balometer capture hood for measuring air flow at vents. The mean value for air entering the animal house has been used, but this may not fully represent airflow into the animal house over the 16 month study period. It is likely that there were fluctuations in airflow with changes in ambient atmospheric pressure, as well as changes in the 'leakiness' of the animal house door despite regular re-sealing, and deterioration over time of the ventilation system extractor fans. The air infiltration into ductwork between the ward (including the day clinic space) and the animal house was 12% (SD 3.5%). 5-10% of air leakage or infiltration is normally accepted by engineers. Despite great efforts working in the false roof of the ward, some ductwork was inaccessible, and thus resealing of all ductwork between the ward and the animal house was not possible. However, this rate of air infiltration was factored into the airborne infection model and so did not adversely affect assessments of relative patient infectiousness. From Figure 4.1 it can be seen that some beds were located closer to air extraction vents than others, and it is likely that air mixing within the room was very different from the perfect mixing assumed by the airborne infection model used to calculate values of relative patient infectiousness. Computational fluid dynamics modelling of half of one of the rooms in the study (i.e. just two beds) predicted concentrations of infectious particles around the room produced by different coughing patients in each of the beds to be far from uniform, with increased concentrations closest to the source.[144] However, without sophisticated tracer gas equipment with multiple sensors located in different parts of the room to measure

local differences in air exchange rates within the room, it is difficult to make accurate adjustments to calculations of infectiousness based on bed location. An additional factor to consider is that the extraction flow rates in the bathrooms were relatively high compared with those at the extraction vents in the walls in the ward spaces themselves. This arose because the length of ductwork between the extractor fans and these bathroom vents situated in the ceiling was shorter than that for the ward extract vents situated at a height of 60 cm from the floor, and through poor design no adjustment had been made in the diameter of these ducts, and no air flow dampers had been installed. Whilst the bathrooms did not have their own air injection, it was found that the windows in the bathrooms had at one point been forced open, such that despite being closed again there was some infiltration of outside air into the bathrooms. These local variations in ward ventilation are also not accounted for in the calculations of patient infectiousness, but this would result in the values of infectiousness presented being underestimates. It should also be noted that there was slightly greater air extraction from one room compared to the other, again due to the absence of any dampers in the ventilation system. This difference has not been factored in to calculations of patient infectiousness.

Due to a lack of an air lock or anteroom at the entrance to the animal house, it was impossible to avoid outside air infiltration into the animal house every time the door was opened, which despite being kept to a minimum occurred at least twice every day. It is also likely there was some further minor outside air infiltration into the animal house around the closed door and around the sealed windows. It was not possible to measure this additional air infiltration accurately, and therefore this further dilution of the air breathed by the guinea pigs is not taken into account in the airborne infection

model, which would again result in the calculations of patient infectivity presented being slight underestimates.

4.4.2 Limitations of the Wells-Riley model of airborne infection

The main limitations of the Wells-Riley model of airborne infection involve the assumptions made in its derivation and use. These include that all susceptible individuals are equally susceptible and have the same pulmonary ventilation rate, that the air in the room space is completely mixed, and that the infectious agent is randomly distributed throughout the room space. From the evidence in the literature, it is reasonable to assume that guinea pigs are highly susceptible to tuberculosis infection[161,180,181] and that whilst susceptibility may vary between strains,[170] it did not vary substantially in the out-bred animals used in this research which were of the same strain and from the same source. As mentioned in the preceding section, mixing of air in the ward was unlikely to be complete, and an infectious agent is likely to be at much higher concentrations close to the source, as predicted in computational fluid dynamics modelling.[144] However, by the time the air reached the animal house through approximately 20 meters of twisting ductwork, it was likely to be well mixed, and using smoke emitters a relatively even distribution of smoke was visualised dispersing throughout the animal house. Another assumption made by the Wells-Riley model is that conditions are under steady state. The model assumes that a steady state concentration of infectious particles has been reached between production of infectious particles by the infector and dilution of these particles by room ventilation. This is rarely the case, as patients tend to cough intermittently, releasing boluses of infectious particles into the air. This is one of the limitations of the Wells-Riley model, and this has been elegantly demonstrated by Beggs and colleagues, where it

was shown that the model predicts higher rates of new infections when the initial quanta level is below steady state, and lower rates of new infections when the initial quanta level is above steady state, as is likely to occur in poorly ventilated spaces.[126]

4.4.3 Significance of incomplete spoligotyping data

As stated in Section 4.3.8, spoligotype results were available for only 49 (42%) of the 118 pulmonary TB admissions. Despite this, 10 (83%) of a minimum of 12 infectious patients were identified, on the basis of matching spoligotype patterns between animals and patients, and patient residence on the ward during an appropriate time period before TB was diagnosed in the guinea pig. It is possible that infection occurred not only from these 12 infectors, but also from other patients on the ward at the same time, for whom spoligotype patterns were not available. This would lead to a greater number of infectors than 12, and to the values of q presented being over-estimates, perhaps significantly so. It is impossible to rule out this possibility; however there are some factors which reduce its likelihood.

Of the ten infectors identified, nine were sputum smear positive. There is a large body of evidence in the literature supporting the fact that the majority of TB transmission occurs from smear positive patients.[78-80] However it should be noted that smear negative transmission should not be underestimated, and in a large study by Behr and colleagues 17% of transmission was estimated to have resulted from smear negative cases.[81] It is possible that TB transmission to the guinea pigs occurred from smear negative patients for whom spoligotype patterns were not available. However, this would require that they were by chance on the ward at a similar time to another patient with a strain with the same spoligotype pattern who had already been

determined as a likely infector. Indeed two groups of guinea pigs (a single and a pair of animals) had patterns which could not be matched to those available for ward patients, and thus presumably were infected by some of these other patients. In this study, 16 patients, of whom four were sputum smear positive, had culture positive sputum, but no spoligotyping result. Fortuitously, these patients were in most cases not resident on the ward at an appropriate time before each of the 10 clusters of guinea pigs with known infectious patient sources were infected. If they were on the ward at the correct time to cause infection in the guinea pigs, these patients had a TB strain of incompatible drug sensitivity, thus excluding them as potential co-infectors. Indeed three of the four sputum smear and culture positive patients with unknown spoligotype patterns were resident on the ward at times corresponding to the infection of the two clusters of guinea pigs with unknown infectious patient sources. With regard to the highly infectious patient responsible for the outbreak in months 10-12, there were no other MDR-TB cases on the ward in the whole period corresponding to the time of infection of these guinea pigs for whom spoligotype patterns were unavailable, making it highly likely that this patient was the sole source of infection.

A further possibility to consider is that guinea pig infections occurred from staff or ward visitors who had tuberculosis. This however is made less probable by the fact that staff would have been likely to seek medical attention early if they developed symptoms of TB, and would not have continued to work on an HIV ward. In addition, all staff and visitors to the ward wore high quality N95 particulate respirators, provided by the hospital in the case of staff and by the study in the case of visitors. It is also possible that infection in the animals occurred from occupants of the small day clinic room from which air was also exhausted over the guinea pigs. It is not possible

to exclude this, but several observations made this less likely. The first observation was that the amount of air exhausted from this room only amounted to approximately 12% of the total air passing over the guinea pigs. Secondly, the patients treated in this room did not have known tuberculosis; thirdly, the same health care workers staffed this area; and finally the room was only used in the mornings.

It is possible that guinea pigs became infected with more than one strain of *M. tuberculosis*, as has been observed in human TB infection.[182,183] Indeed continuous exposure to ward air potentially contaminated by multiple TB patients on the ward would perhaps make infection with multiple strains likely. However, in the 10 guinea pigs in which three separate tissues were cultured, and in the nine animals in which two separate cultures of tissues were performed, only one spoligotype pattern was ever seen in each guinea pig. In the nine animals with multiple primary pulmonary foci in which foci were dissected and cultured separately, again only one spoligotype pattern was observed in each animal, suggesting monoclonal infection in these animals.

4.4.4 Spatial distribution of disease: evidence for airborne and not horizontal spread of disease

Evidence is presented in Figure 4.9 and Figure 4.10 for the conclusion that TB infection in the guinea pigs occurred via the airborne route from patients on the ward rather than via horizontal spread between animals. Guinea pig TB infections were randomly distributed throughout the animal house, and infections with TB strains with the same spoligotype pattern occurred at similar time points on either side of the central partition. The patient responsible for the month 10 outbreak (26th March 2003

skin test) was resident on the ward from 22nd February – 26th March 2003. To have caused infection in animals such that their PPD skin test reactions were detected as positive in the March 26th and April 27th skin test is therefore entirely reasonable. To have caused infection in the six animals with the same spoligotype pattern in the May 31st skin test would imply an 'incubation' period in these animals of at least 33 days (from the last day on the ward of the infectious patient to the day after the skin test of April 27th when these six animals still demonstrated a negative PPD skin test). This is slightly longer than the 21 days reported in the literature for guinea pigs exposed to TB aerosols to develop a positive PPD skin test, concomitant with haematogenous spread of *M. tuberculosis* organisms after passage from the pulmonary primary focus to the broncho-hilar lymph nodes.[184] However, these studies involved fully virulent H37Rv strains of *M. tuberculosis*, and guinea pigs of a different variety to the Peruvian animals used in this study. It may be that some strains of *M. tuberculosis*, in particular drug-resistant strains, require a longer period of incubation in guinea pigs that may be up to six weeks (personal communication Dr D McMurray). Riley cited a 3-4 week incubation period for TB in guinea pigs, and thus determined that the ward occupancy for a patient to infect a guinea pig would be 3-7 weeks before the corresponding infected guinea pig became PPD positive.[75,76]

For the animals that were PPD positive in the May 31st skin test to have been infected horizontally by other animals with the same TB strain, as detailed in Section 4.3.12, it would have been necessary for the following conditions to have been met: animals on both sides of the central partition and in cages 'upwind' of the six which were PPD positive in May must have become infected with TB early during the stay of the infectious patient on the ward, been PPD false negatives in the March skin test, and

have rapidly developed disseminated disease such that it could be transmitted by the faecal-oral route to other animals in their own cage, or by the airborne route to cages 'downwind.' This is unlikely, supported by the work of Lurie which showed airborne transmission between guinea pigs only after extended periods with disease,[174] and also by the work of Riley: in the second two years of his study period he introduced animals that had been deliberately infected with TB by the airborne route no more than three weeks earlier (and which were therefore still PPD skin test negative) into a second exposure chamber which was protected by high dose ultraviolet irradiation in the ducts of all air entering the chamber.[76] These animals were skin tested a month later (by which time they were now positive) and removed. None of the other animals in the chamber went on to develop TB infection, either from the ward (protected by ultraviolet irradiation air in ducts) or from the tuberculous guinea pigs with which they had shared cages for a month, presumably because the infection in the tuberculous animals was not sufficiently advanced to be infectious horizontally. Therefore it appears likely that the 6 animals PPD positive in the month 12 (May 31st) skin test with the same spoligotype pattern as the month 10 outbreak were infected by the same patient. With a delay of up to 33 days from infection to the development of a positive PPD skin test, ward occupancy for a patient to infect a guinea pig in our study was 3-9 weeks before the corresponding infected guinea pig became PPD positive.

Further evidence for the airborne spread of disease is provided by the fact that all guinea pigs had evidence of pulmonary TB, suggesting the arrival of an infectious droplet nucleus by the airborne route, rather than horizontal transmission by the faecal-oral route. Specifically, a primary focus in a lung lobe and/or tuberculous involvement of broncho-hilar lymph nodes would suggest the airborne route, whereas

a pattern of disease involving cervical and mesenteric lymph nodes would suggest an oral route of transmission.[174] Only two guinea pigs had evidence of mesenteric involvement raising the possibility of faecal-oral spread of disease. These two guinea pigs also had autopsy evidence of airborne TB (they were *PPD* positive in month *10)* and they had spent several months in cages with other tuberculous animals before autopsy, without wire mesh cage floors that allow drop through of faeces. It is likely their intestinal TB represented horizontal transmission from these other animals in the cages after having been removed from the exposure chamber rather than horizontal transmission in the exposure chamber itself. However, in spite of this, the animal exposure chamber was re-designed and re-built with a transverse rather than a longitudinal pattern of air flow to minimise even the theoretical risk of horizontal spread of tuberculosis between animals for subsequent experiments.

4.4.5 Relative infectiousness of patients

In the light of the evidence presented above, it is likely that guinea pig infections occurred from 12 infectious patients out of a total of 97 patients responsible for 118 ward admissions. TB transmission therefore occurred from a minority of patients, and within the group of 12 infectious patients there was considerable variability in infectiousness, as measured by values for q. It should be pointed out that these are values of q calculated for TB transmission from patients to guinea pigs, and guinea pigs may have increased susceptibility to TB than humans. Thus some caution is needed in comparing the infectiousness of these patients by values for q calculated in this thesis with those values for q calculated for human to human transmission in the literature, such as the office worker[125] and the bronchoscopy case.[7,123] However, useful comparisons can be drawn with values of q calculated for the patients studied

by Riley, q=1.3 for all patients averaged over the four years of the study, or q=60 for the highly infectious case of laryngeal TB. Whilst the infectious dose of *M. tuberculosis* for humans is not known, hence the concept of infectious quanta used in models of airborne infection, there is evidence that whilst just one droplet nucleus is necessary to establish infection in guinea pigs with fully virulent strains of *M. tuberculosis*, with strains of reduced virulence for guinea pigs, up to four aerosolised colony forming units may be required to produce a primary focus of TB in the lungs of a guinea pig.[104]

Good evidence is presented for the presence of a highly infectious patient infected with MDR-TB. At least 108 guinea pigs were infected by this patient, and a further 15 animals were infected in the same period, but for which TB strain spoligotype patterns were unavailable. If 108 animals are considered as new cases, q for this patient was 281, but if the 15 other animals are also included as new cases, q increases to 357. These values of q are in excess of the value of 60 calculated for the laryngeal case of TB in Riley's study (human to guinea pig transmission), and the value of 250 (human to human transmission) reported for a large TB outbreak associated with intubation and bronchoscopy of a TB patient, procedures likely to induce considerable generation of infectious aerosols. This spoligotype pattern was only observed in three ward patients out of a total of 39 for whom spoligotyping was available (in which 23 patterns were observed in total). The second patient, who caused infection in the guinea pigs, is discussed below. The third patient was sputum culture negative, and was only on the ward for a three day period, at the end of month 14 of the study, and therefore too late to be responsible for any of the guinea pig infections in the second, smaller outbreak with this strain seen in months 14 and 15.

The second patient infected with a TB strain with this same spoligotype pattern was admitted to the ward in month 13, and was also highly infectious, q=64, resulting in guinea pig infections in months 14 and 15 of the study. The appearance of two outbreaks associated with this MDR strain suggests some strain related factor involved in transmissibility, for example the ability to survive the process of aerosolisation and the stresses of desiccation and exposure to light whilst suspended in the air as a droplet nucleus. An alternative explanation would be an effect of this strain on disease phenotype. It is interesting to observe that both patients with this strain produced large volumes of sputum, between 10-90 ml during 24 hour periods. Neither patient had evidence of cavitation on chest X-ray. Though there was no documentation of a formal ear-nose-throat assessment, neither patient was recorded in their medical histories to have had laryngeal TB. Both patients also spent a period on the ward untreated. The q=281 patient was untreated for the first 11 days of a 32 day admission before treatment with second line drugs was commenced. The delay was caused by difficulties with the availability of second line medications. The q=64 patient had recently been commenced on a sub-optimal treatment regimen ('Esquema 2', standard first line therapy with the addition of streptomycin)[4] and this was suspended for 10 days of the 26 day ward admission owing to adverse effects of the medications.

In the univariate analysis of 49 admissions for which spoligotype patterns were available, sputum smear positivity, MDR-TB *vs.* drug-sensitive TB and sub-optimal treatment were significantly associated with TB transmission to the guinea pigs ($p \leq 0.05$). The association of sputum smear positivity with TB transmission concurs with the evidence in the literature about the infectiousness of smear positive

patients.[78-80] As discussed in the introduction, there is conflicting evidence for the transmissibility of MDR *vs.* drug-sensitive TB strains.[105-111] Despite the fact that patients with drug-sensitive TB strains were responsible for 55% of total pulmonary patient days, they accounted for only 3 (25%) of the 12 ward admissions resulting in TB transmission to the guinea pigs. However, the significant association of MDR-TB *vs.* drug-sensitive TB with TB transmission in this study should be viewed with caution because there was co-linearity with another variable significantly associated in univariate regression with TB transmission from ward to guinea pigs, namely sub-optimal treatment ($p=0.02$). Five out of the six identified infectious ward admissions with MDR-TB were being treated with sub-optimal regimens, and one had had this treatment temporarily suspended owing to adverse effects. Information about the drug treatment of 10 out of 12 infectious patients was available. It is noteworthy that all of these 10 infectious patients had only recently started treatment during or within two weeks of the ward admission when they infected guinea pigs, or had had their treatment suspended due to adverse effects, or were drug-resistant cases on sub-optimal treatment regimens. This concurs with the evidence in the literature about the effect of effective anti-tuberculous chemotherapy on patient infectiousness.[75,80]

The incomplete set of spoligotype patterns for the patients (49 of 118 admissions) and the subsequent failure to identify two of the minimum of 12 infectious source patients weakened the analysis of the determinants of infectiousness. Spoligotype patterns were available for a further eight admissions (57 of 118 in total) if patient sputum samples collected up to one year prior to their ward admission were considered. In univariate analysis of this larger set of data, the same three factors (sputum smear positivity, MDR *vs.* drug-sensitive TB, and sub-optimal treatment) remained

significantly associated with TB transmission to the guinea pigs ($p \leq 0.05$). Patient consent to enter the study was disappointingly low, despite the relatively modest level of participation required. This poor uptake of patients into the study further hindered the analysis of patient determinants of infectiousness such as cough frequency, haemoptysis and sputum production as data from only 33 admissions was available for analysis, and it is likely that multiple factors related to both the host and the TB strain are important in determining the infectiousness of TB patients.

4.4.6 Conclusion

This chapter has presented data including guinea pig and patient TB strain drug sensitivity patterns corroborated by molecular fingerprinting to offer further evidence that guinea pig TB infections arose from patients on the ward via the airborne route. Guinea pig TB infections fell into twelve clusters based on spoligotype patterns, and it is probable that each cluster was associated with one infectious patient owing to the temporal association of guinea pig infections and the ward admission of patients infected with TB strains with identical spoligotype patterns. Through use of the Wells-Riley model of airborne infection, relative values of infectiousness (q, infectious quanta produced per hour) were calculated for these twelve infectious patients. Drug-resistant cases were responsible for the great majority of guinea pig infections, and there was good evidence for the existence of a highly infectious patient, whose q value was 281, in excess of the q=60 for the most infectious case observed by Riley during the four years of his study, a case of laryngeal TB. Perhaps one of the most important findings was that six of the seven identified infectious patients with drug-resistant strains were on treatment regimens that were sub-optimal. This gives weight to the importance of culture based diagnosis for TB and early drug sensitivity testing in

national TB programmes, and prompt initiation of effective second line anti-tuberculous chemotherapy to prevent ongoing TB transmission.

Table 4-1: Anti-tuberculous chemotherapy treatment status of confirmed and presumed drug-sensitive and drug-resistant TB admissions to the ward

Number of days on the ward are shown, with percentage of total pulmonary TB days (1798 days) in brackets, for those patients on established treatment (≥2 weeks duration); recent treatment (commenced within the last 2 weeks); new treatment (commenced during admission); or those off treatment (abandoned or withdrawn due to adverse effects). Treatment is further categorised as 'correct' or 'sub-optimal' (see text for details).

	DRUG-SENSITIVE TB PATIENT ADMISSIONS			DRUG-RESISTANT TB PATIENT ADMISSIONS		
Treatment	Admis-sions	Days on ward	Days without treatment	Admis-sions	Days on ward	Days without treatment
Established correct	39	492 (27%)	19 (1%)	8	115(6%)	0
Established sub-optimal	-	-	-	8	115 (6%)	0
Recent correct	5	84 (5%)	0	4	100 (6%)	0
Recent sub-optimal	-	-	-	7	159 (9%)	0
New correct	19	323 (18%)	77 (4%)	5	93 (5%)	32 (2%)
New sub-optimal	-	-	-	6	121 (7%)	29 (2%)
None	4	63 (4%)	63 (4%)	9	78 (4%)	78 (4%)
Unknown	-	-	-	3	47 (3%)	-
Total	67	962 (54%)	164 (9%)	50	828 (46%)	139 (8%)

Table 4.2: Guinea pig TB strain drug resistance and spoligotype patterns

Drug sensitivity is shown for isoniazid (INH), rifampicin (RIF), streptomycin (SM), ethambutol (EMB), capreomycin (CAP) and ciprofloxacin (CIP) for the 12 clusters of guinea pigs according to their spoligotype pattern and approximate date of acquiring infection. Drug sensitivity results were obtained using the TEMA assay.[165] (* denotes the 52 guinea pigs from the month 10-12 outbreak of 108 animals with the same spoligotype pattern for which drug sensitivity results were available).

Cluster	Guinea pig #	MIC µg/ml						Drug resistance	Spoligo-type strain #
		INH	RIF	SM	EMB	CAP	CIP		
1	133-N	R	R	R	R	S	S	MDR	1
2	243-B	S	S	S	S	S	S	S	2
3	247, 265-N	R	R	R	R	S	S	MDR	3
4	268, 271-N	R	R	S	R	S	S	MDR	4
5	259-N	S	S	S	S	S	S	S	5
6	289-N	R	R	S	S	S	S	MDR	6
7	286-N	R	R	R	R	S	S	MDR	1
8	397-660 N*	R	R	S	R	S	S	MDR	7
9	614-N	R	S	S	S	S	S	DR	8
10	608, 635-N	R	R	S	R	S	S	MDR	3
11	666-N	S	S	S	S	S	S	S	5
12	752,736,671 N, 675-P	n/a	n/a	n/a	n/a	n/a	n/a	presumed MDR	7

Table 4.3: Characteristics of the 12 infectious patients

Characteristics are shown of the 10 identified and two unidentified infectious patients identified by spoligotype and temporal association with a guinea pig infection. It is notable that of the 10 identified infectious patients, nine were smear positive. Two of the three drug-sensitive cases were newly started on treatment and hence spent time on the ward untreated, and the third had stopped treatment. Six of the seven identified infectious drug-resistant cases were on sub-optimal treatment regimens.

Patient #	Days on ward	Sputum smear	Drug sensitivity	Treatment	Days on ward off treat-ment	Number of guinea pigs infected	Spoligo -type pattern #	Calculated infectious quanta per hour
1	6	3+	MDR	Recent sub-optimal	0	1	1	15 (14-15)
2	24	1+	Sensitive	New	3	1	2	3.7 (3.6-3.9)
3	39	2+	MDR	New sub-optimal	5	2	3	3.6 (3.4-3.7)
4	?	?	MDR	?	?	2	4	?
5	13	2+	Sensitive	New	1	1	5	6.9 (6.7-7.1)
6	43	3+	MDR	Recent sub-optimal	0	1	6	2.3 (2.2-2.4)
7	?	?	MDR	?	?	1	1	?
8	32	3+	MDR	New correct	11	108	7	281 (272-291)
9	7	3+	Isoniazid resistant	Recent sub-optimal	0	1	8	23 (22-24)
10	13	3+	MDR	Established sub-optimal	0	2	3	50 (48-52)
11	18	Negative	Sensitive	Stopped	18	1	5	16 (15-16)
12	21	3+	MDR	Recent sub-optimal	10	4	7	64 (62-66)

Table 4.4: Determinants of infectiousness

Univariate regression analysis was performed on putative determinants of infectiousness for the 49 ward admissions by pulmonary TB patients for which spoligotype patterns were available. For categorical independent variables, the number of cases (% of total in brackets) is shown, and for continuous independent variables, the median (inter-quartile range in brackets) is shown.

Independent variable	Dependent variable		Univariate logistic regression	
	Infectious patient admissions n=10	Non-infectious patient admissions n=39	Co-efficient (95% CI)	p
Age	31 (27-43)	31 (27-39)	1.0 (1.0-1.1)	0.4
Male sex	9 (90%)	29 (74%)	0.32 (0.0-2.9)	0.3
Days on ward	20 (12-34)	14 (7-25)	1.0 (1.0-1.1)	0.4
Sputum smear positive	9 (90%)	20 (51%)	0.1 (0.0-1.0)	0.05
MDR-TB *vs.* drug-sensitive TB	6/9 (67%)	6/31 (19%)	0.1 (0.0-0.6)	0.01
Isoniazid mono-resistant *vs.* drug-sensitive TB	1/4 (25%)	6/33 (18%)	0.7 (0.1-8.2)	0.8
Sub-optimal treatment	7 (70%)	11 (28%)	5.9 (1.3-27)	0.02
Days on ward without treatment	0 (0-6)	0 (0-2)	1.0 (0.9-1.1)	0.7

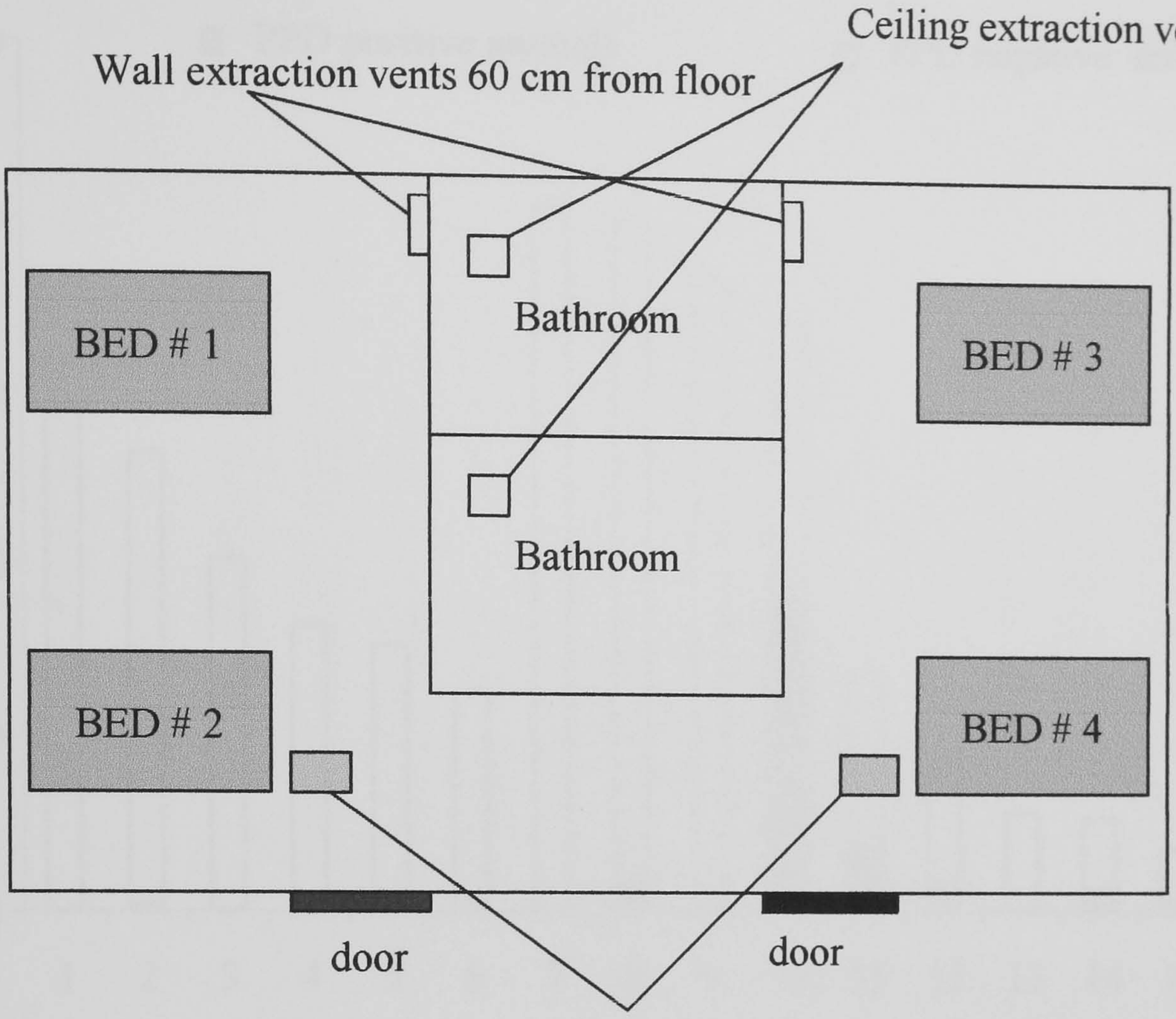


Figure 4-1: Layout and ventilation system of four-bedded patient room

The second four-bedded patient room was identical in its design and layout. Unfiltered outside air entered through two ceiling injection vents, and was extracted through two 6" x 6" ceiling extraction vents in the bathrooms, and two 12" x 12" extraction vents in the wall situated 60 cm from the ground.

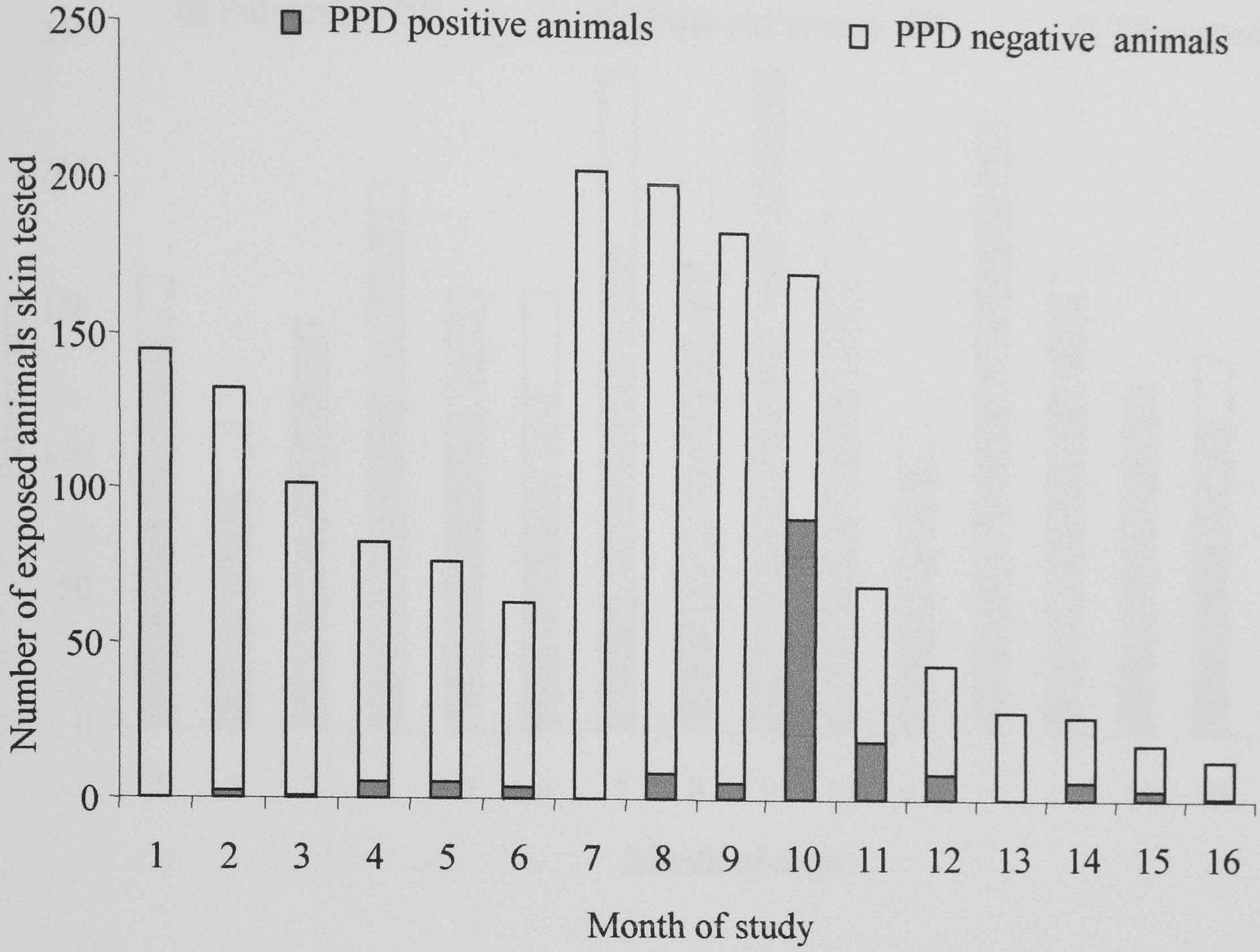


Figure 4-2: Total numbers of exposed guinea pigs and number of PPD positive animals at each monthly skin test

Total number of exposed guinea pigs PPD skin tested for each month of the study, the month 1 test corresponding to 20th June 2002. The grey shading represents the numbers of animals that were PPD skin test positive (i.e. induration ≥7.5 mm at 24 or 48 hours) for that monthly test. The difference in total numbers of guinea pigs between months reflects the removal from the exposure chamber of PPD positive reactors for sacrifice, the removal of exposed PPD negative controls, and intercurrent deaths. An unusually large number of PPD positive animals were seen in month 10.

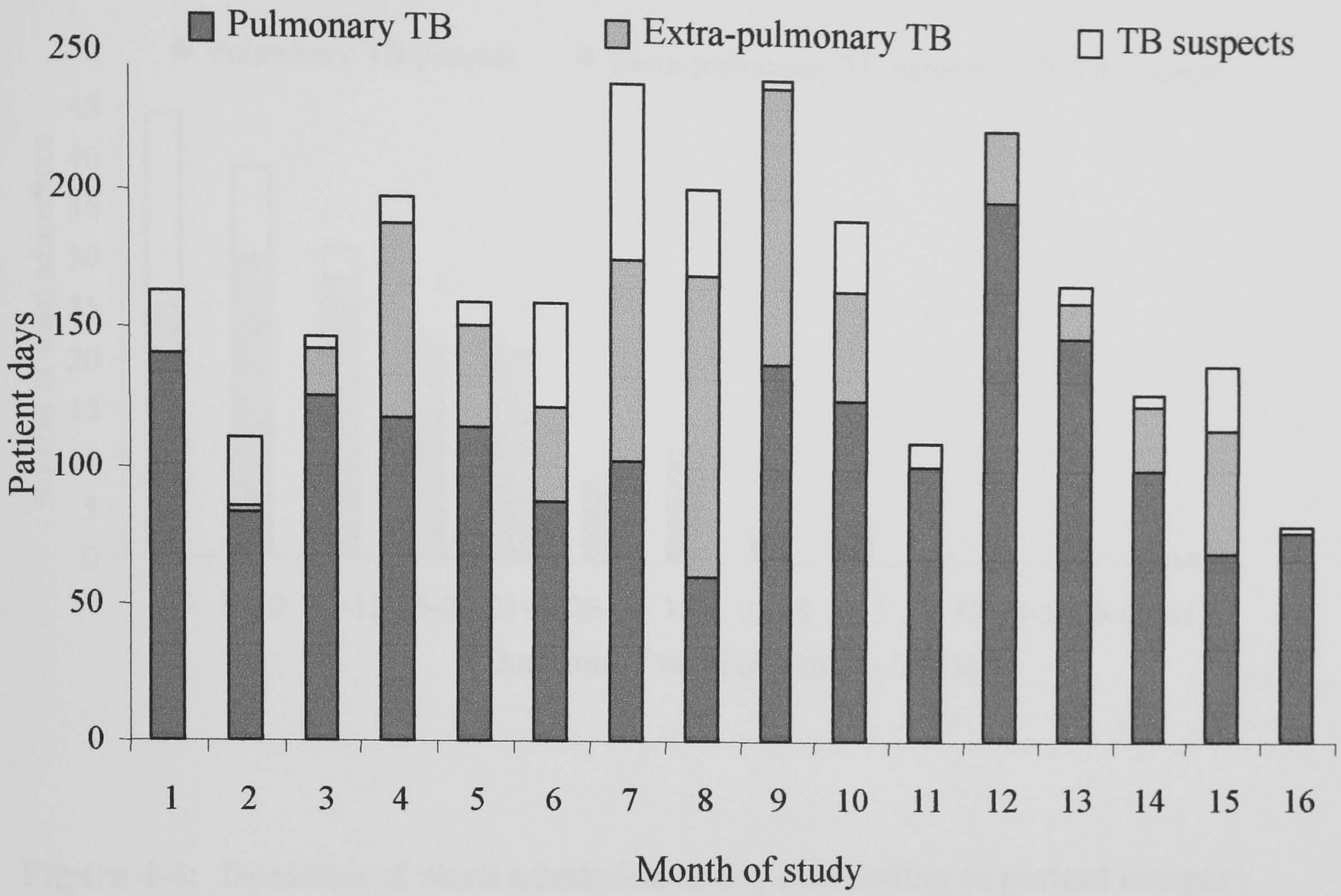


Figure 4-3: Bed occupancy by month of study according to patient category

There were total 2667 patient days of a total possible 4040, resulting in average bed occupancy of 66% for the 8 beds in the study. There were 185 ward admissions by a total of 161 patients, of whom 97 had pulmonary TB, 30 had extra-pulmonary TB, and 34 were TB suspects who were subsequently found to have no laboratory evidence of TB.

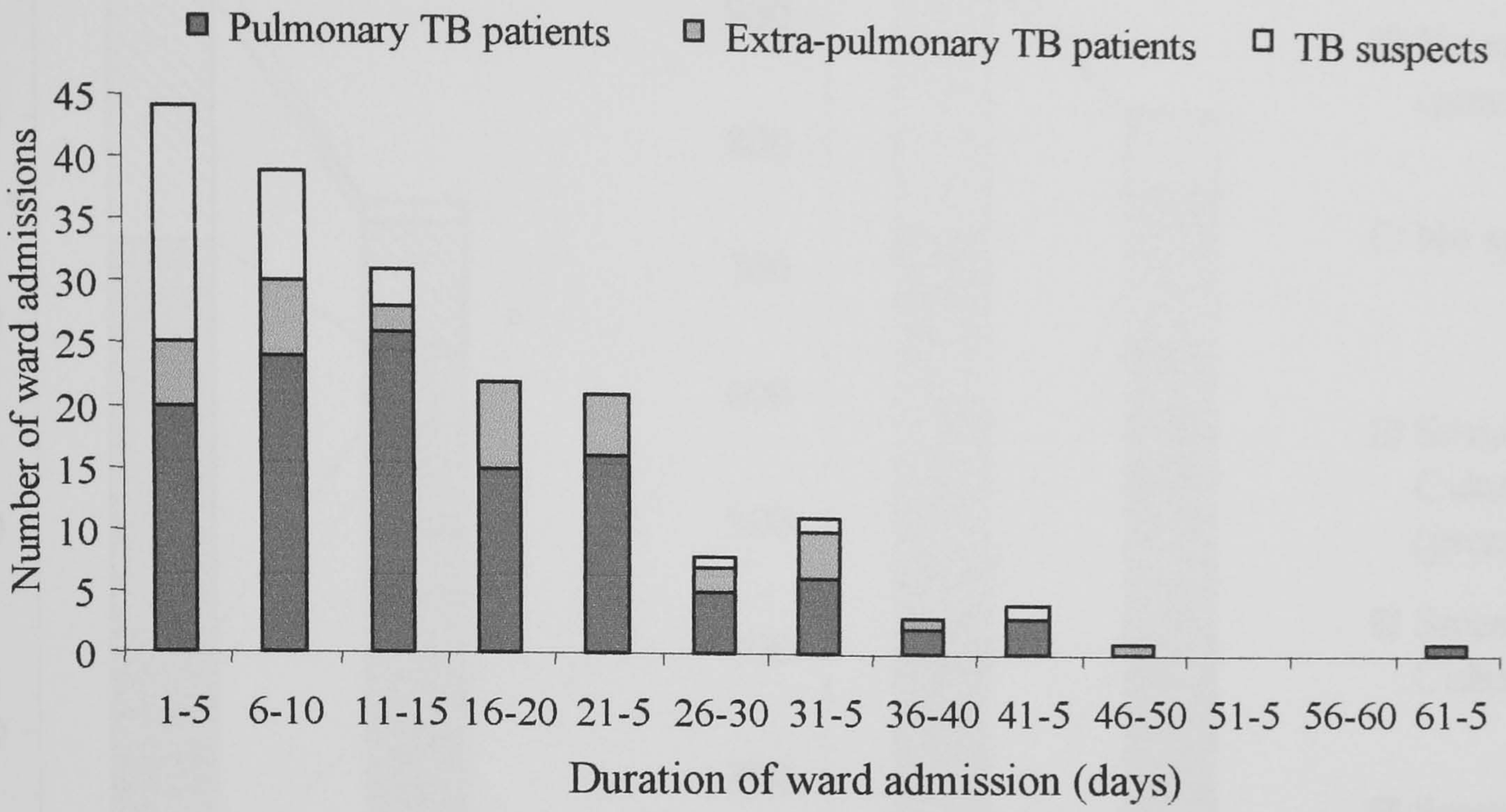
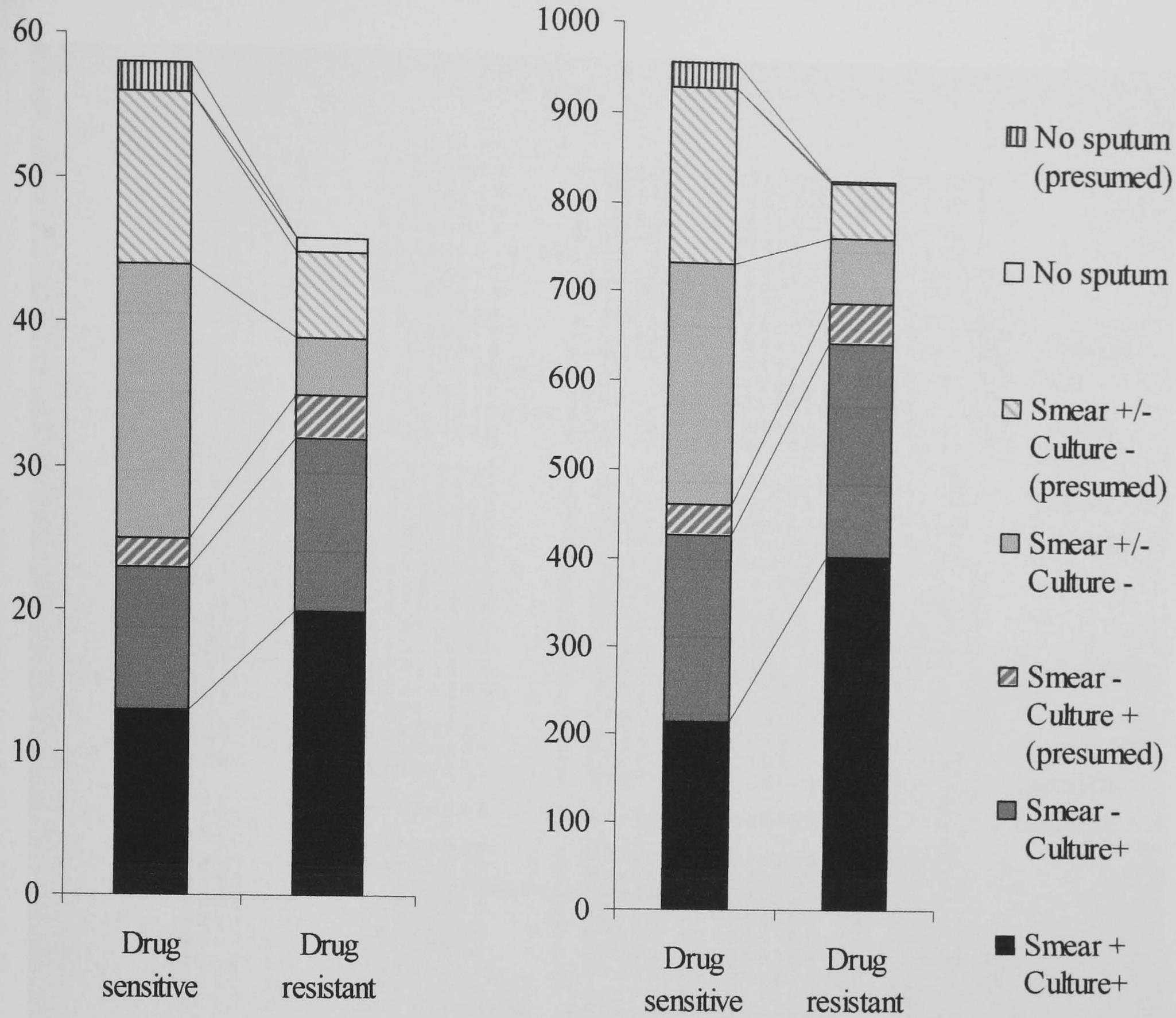


Figure 4-4: Duration of ward admission in days according to patient category

The median length stay on the ward for all patients during the study period was 11 days (inter-quartile range 6-21 days). Median duration of ward admission was 13 days (7-22) for pulmonary TB patients; 18 days (9-26) for extra-pulmonary TB patients; and 5 days (2-9) for TB suspects. The three TB suspects with long ward admissions >25 days include two patients treated empirically for TB, and one patient who had just completed a course of anti-tuberculous chemotherapy a week prior to admission.



(i) Number of cases **(ii) Patient days**

Figure 4-5: Pulmonary TB patients on the ward according to sputum smear status and TB drug sensitivity: (i) Number of cases (ii) Number of patient days

The total number of cases shown in the left hand graph (i) is 104, attributed to 95 patients. This reflects the three patients with drug-sensitive TB who acquired MDR-TB during the study and also some patients with multiple admissions where smear or culture status changed. Two patients are not included in these figures: one culture positive patient with drug-sensitive TB (accounting for 6 patient days) with an unavailable smear result; and one smear negative, culture positive patient (accounting for 8 patient days) with no TB drug sensitivity result. 'Presumed' denotes empirical results for drug sensitivity where microbiological confirmation was unavailable.

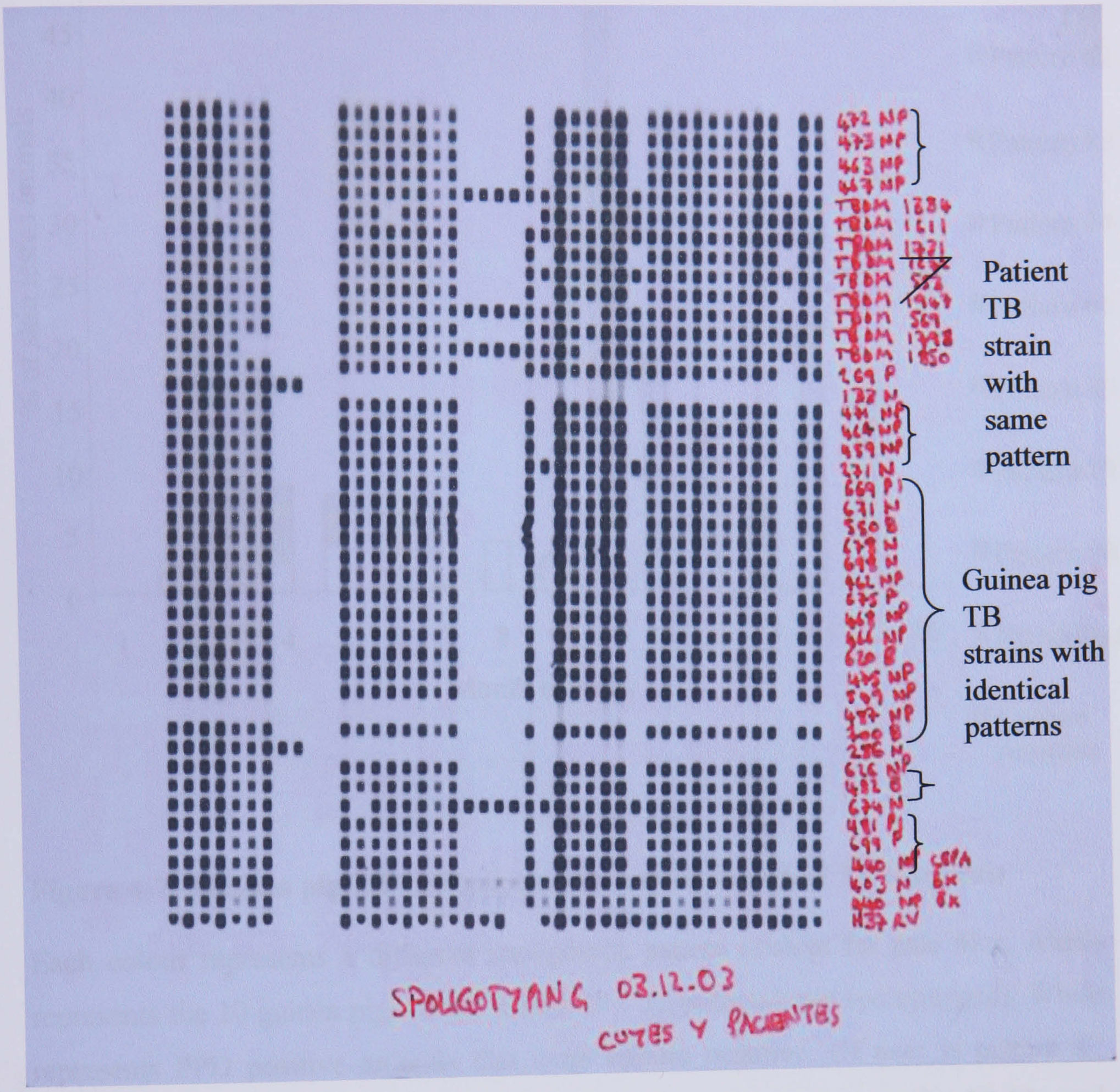


Figure 4-6: Example of a spoligotype film of guinea pig and patient TB strains

Each row corresponds to one strain of *M. tuberculosis*. Each row has 43 squares, and a positive square (black) represents the hybridisation to the spoligotype membrane of PCR products from the TB strain. The bottom row is a control H37Rv strain of *M. tuberculosis*. The brackets denote guinea pig TB strains from the monoclonal outbreak in month 10. Also seen on this film are two samples from the same patient with an identical spoligotype pattern to that of the monoclonal outbreak in guinea pigs.

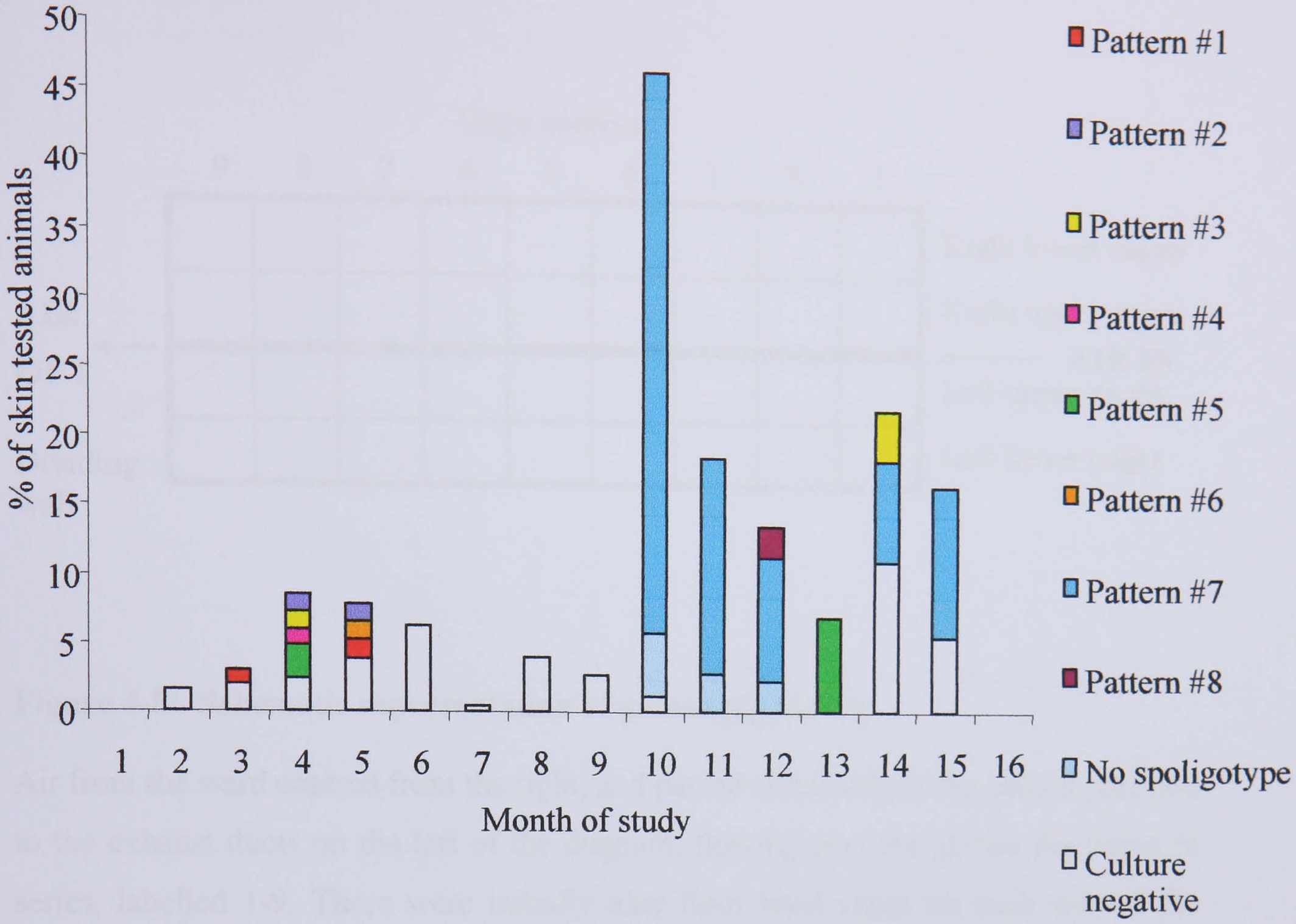


Figure 4-7: Guinea pig TB spoligotype patterns by month of TB diagnosis

Each colour represents a different spoligotype pattern (except for pale blue, which represents the 10 guinea pigs of the month 10 – 12 outbreak not spoligotyped). White represents PPD positive animals that were culture negative. Of note is pattern #7 (coloured turquoise) which was seen in a large outbreak in months 10-12, and appeared again in another outbreak in months 14-15. Also of note is the appearance in separate months of the patterns #1, #3, and #5, colour coded red, yellow and green respectively.

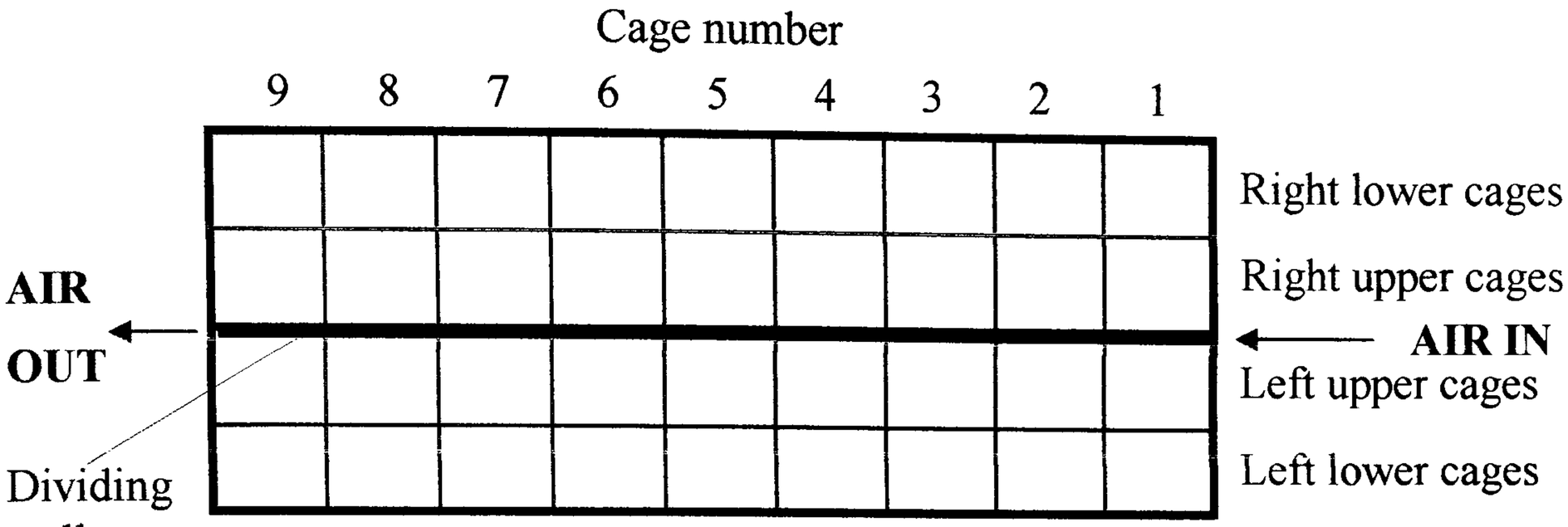
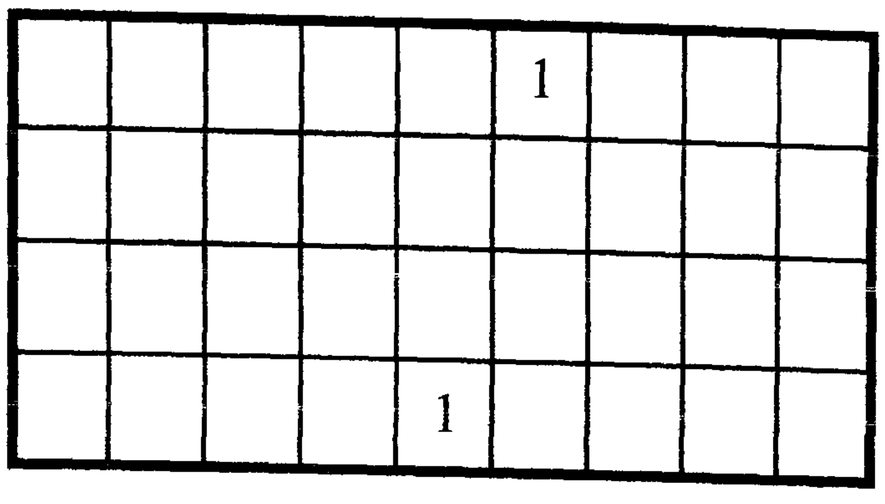
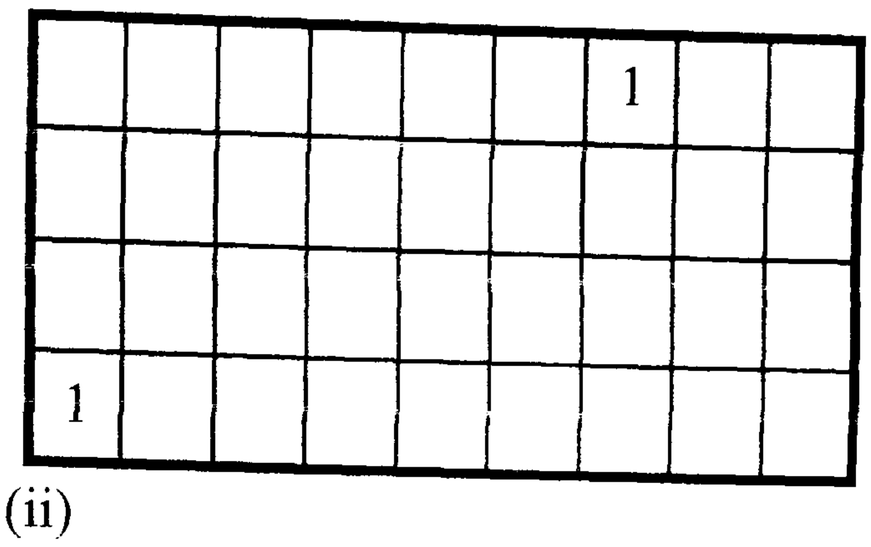


Figure 4-8: Schematic representation of guinea pig facility

Air from the ward entered from the right, and passed either side of the central partition to the exhaust ducts on the left of the diagram, flowing over the guinea pig cages in series, labelled 1-9. There were initially nine floor level cages on each side of the partition, and an additional nine upper level cages were added in month 6.



(i)



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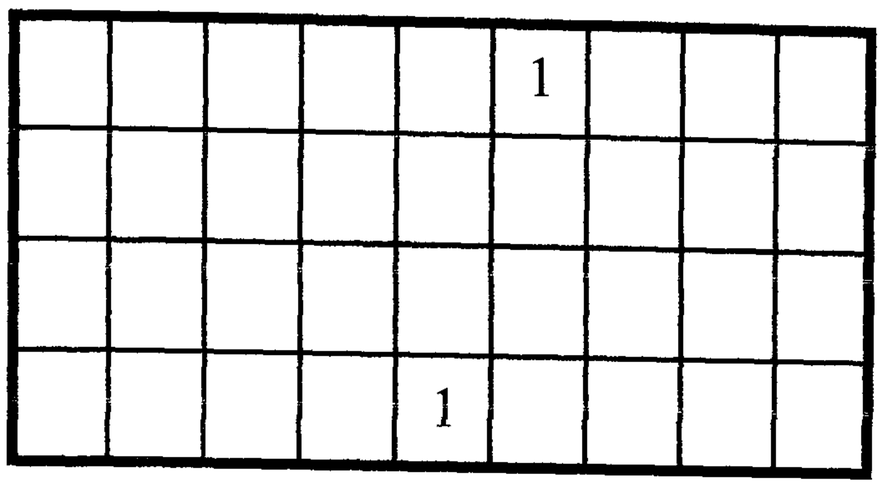

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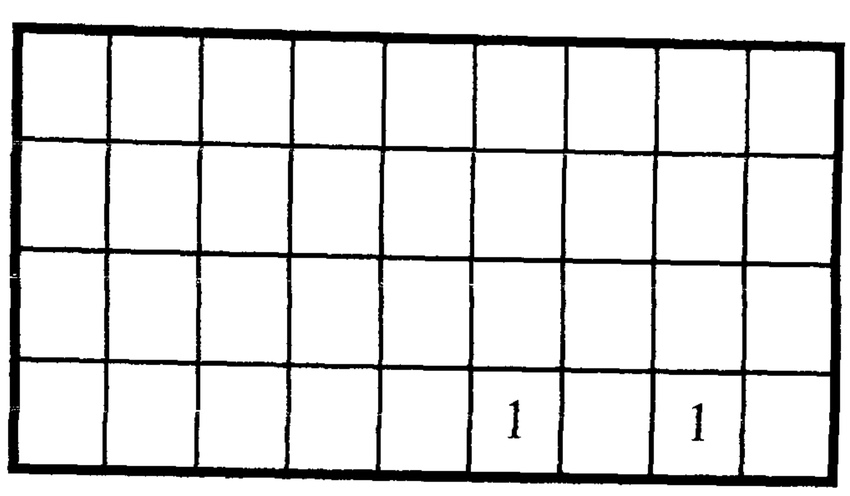

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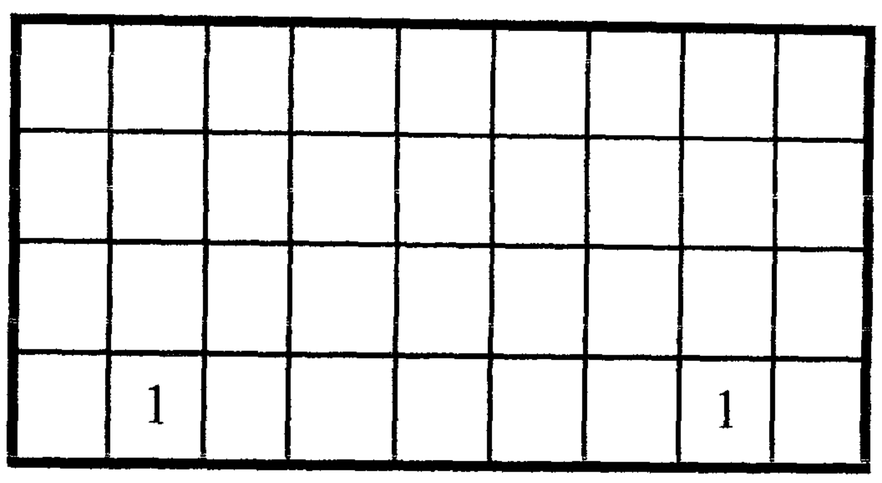

(v)

Figure 4-9: Spatial distribution of guinea pig infections with identical spoligotype patterns - evidence for airborne and not horizontal spread of TB

In figures (i) to (iii) infection (shown by numbers of animals infected) occurred either side of the central partition. In figures (iv) and (v) infection occurred on the same side of the partition, one cage therefore being 'downwind' of the other, but infection occurred at similar time points. This makes horizontal spread unlikely because of the minimum three week incubation period in the 'index case' which would be required.

(i)

5/5 100%	4/5 80%	2/6 33%	2/4 50%	2/4 50%	0/6	4/7 57%	5/6 83%	5/5 100%
2/6 33%	3/5 60%	0/5	2/4 50%	2/4 50%	1/5 20%	2/4 50%	3/5 60%	4/4 100%
2/3 33%	2/3 66%	4/5 80%	0/4	0/5	2/4 50%	5/6 83%	3/5 60%	1/4 25%
2/7 29%	4/4 100%	4/5 80%	3/6 50%	2/4 50%	2/4 50%	3/4 75%	3/4 75%	4/5 80%

(ii)

		1/3 33%	0/1	1/2 50%	1/4 25%	2/2 50%	0/1	
0/3	0/2	0/4	2/2 100%	0/2	2/2 100%	1/1 100%	1/2 50%	
0/2	1/1 100%	1/2 50%	1/2 50%	0/5	0/3		1/1 100%	1/2 50%
3/3 100%		0/1	1/3 33%	0/2	0/2	0/1	0/1	1/2 50%

(iii)

		0/2	0/1	0/1	0/3		0/1	
1/3 33%	1/2 50%	2/4 50%		1/2 50%			0/1	
1/2 50%	0/1	1/1 100%	0/1	0/5	0/3			1/1 100%
		0/1	0/2	0/2	0/2	0/1	0/1	0/1

Figure 4-10: Spatial distribution of guinea pig infections with identical spoligotype patterns in (i) month 10 (ii) month 11 and (iii) month 12

For each cage, the number of positives is shown, above a denominator which is the total number of guinea pigs in that cage. Also shown is the percentage of animals in the cage with a positive skin test for that month. For each of these three months a random distribution of infection consistent with airborne transmission of disease from the ward was seen. Infections occurred on both sides of the central partition, in both upper and lower cages, consistent with airborne spread of infection from the ward.

CHAPTER 5

General discussion

5.1 Overview

The work described in this thesis is concerned with the prevention of institutional airborne tuberculosis transmission using environmental control measures. In chapter 2 the rates and determinants of natural ventilation by simply opening windows and doors in health care facilities have been described for the first time. The potential effect of this natural ventilation on tuberculosis transmission was investigated using a standard airborne infection model. Significant reductions in tuberculosis transmission compared with mechanically ventilated facilities were predicted. However natural ventilation is not suited to cold climates and especially at night wards may have all their windows tightly shut. There is therefore a need to validate alternative existing environmental controls for TB transmission and to investigate novel interventions. In order to avoid reliance on mathematical modelling, optimal evaluation of environmental controls to prevent TB transmission involves measuring transmission itself. However, detecting airborne TB transmission to health care workers is confounded by exposures outside the workplace and is made logistically difficult by staff turnover which may be high. Detecting viable airborne *M. tuberculosis* droplet

nuclei using mechanical air sampling techniques is difficult.[153] For these reasons a guinea pig air sampling facility above a TB ward was created, based on classic studies from the 1950-60s,[75,76,185] and the optimisation of this model has been described in chapter 3. By using modern molecular tools it has been possible to demonstrate the origin of the transmission of the majority of the tuberculosis detected in the guinea pigs, and this has been described in chapter 4. This has provided novel insights into the infectiousness of TB patients co-infected with HIV, in today's era of MDR-TB. The guinea pig air sampling facility has now been re-designed and expanded, and is currently in use evaluating low-cost, low-technology strategies to reduce airborne TB transmission applicable to resource limited settings where the burden of TB is highest.

5.2 Natural ventilation

Natural ventilation by simply opening windows and doors has been demonstrated to provide high rates of ventilation even on relatively windless days. Natural ventilation was highest in wards of old-fashioned design, with high ceilings and large windows. Opening windows and doors resulted in ventilation rates significantly greater than those provided by mechanical ventilation systems for high-risk settings.

The World Health Organisation recommends natural ventilation as an environmental control against TB transmission in health care facilities in low resource settings.[40] The work presented in this thesis provides the first evidence base for these recommendations. There is a danger that valuable resources may be consumed by the installation of mechanical ventilation systems as low resource countries attempt to attain standards set by guidelines such as those published by the CDC,[112,113] which are

used for reference in many parts of the world. The original 1994 CDC guidelines were recently revised,[113] and do not mention natural ventilation as an environmental control measure, perhaps due to the lack of published evidence. As described in the literature review in this thesis, mechanical ventilation systems frequently fail to deliver the rates of ventilation or directional air flow for which they were designed, often due to inadequate maintenance, and such failings have been implicated in numerous outbreaks of TB. Whilst well maintained negative pressure isolation rooms are undoubtedly protective against TB transmission in health care facilities, their high cost precludes use in much of the world where the prevalence of TB is highest, careful ongoing maintenance is required, and perhaps most importantly, they are limited to certain high risk areas. Institutional transmission of tuberculosis does not only occur in high risk areas, but takes place in emergency departments, out-patient clinics, general medical wards and waiting rooms. These areas are ventilated at much lower rates of air exchange, without negative pressure, and patients are not in isolation. Indeed the patients most commonly found in these areas, undiagnosed and untreated, are likely to be among the most infectious.[76,88] Natural ventilation, being applicable across a wide range of healthcare facility settings, as well as in other institutional settings, therefore offers significant advantages over mechanical ventilation for the control of airborne TB transmission, particularly in low resource settings. Whilst not suited to cold regions, in temperate or tropical climates with a high prevalence of airborne nosocomial disease such as tuberculosis, it may be better for patients, visitors and staff to wear extra clothing in breezy, open-windowed, naturally ventilated wards and waiting rooms than to be warm in stuffy, low-ceilinged rooms as they catch tuberculosis or other airborne diseases.

5.3 Guinea pig air-sampling model and infectiousness of HIV-TB patients

The classical studies of Riley and colleagues[75,76,88] have been successfully re-created and developed using modern molecular methods. In comparison with the non-HIV patients studied by Riley in the 1950s-60s, the heterogeneous mix of HIV-TB patients in this study produced on average six times as many infectious particles. However, if the highly infectious MDR-TB patient identified in this work is removed from the analysis, the average rate of production of infectious particles (q) was 1.2 per hour, almost identical to that calculated for the patients studied by Riley. Whilst those patients included some who had their treatment delayed for study purposes, the patients in this thesis included some with MDR-TB who were sub-optimally treated. The great variability of infectiousness between patients observed by Riley was also seen in this study, and it is interesting to note that the two most infectious patients in this study had a strain of *M. tuberculosis* with an identical spoligotype pattern. This suggests that some strain related factor may be associated with infectiousness and this will be the focus of some future work. Due to the variable duration of the TB incubation period in the Peruvian guinea pigs studied, it is difficult using this model to investigate the duration patient infectiousness after the initiation of treatment. However, new techniques such as Fennelly's cough box[185] are being developed to measure patient infectiousness and it may be possible to validate such techniques against the logistically difficult but currently optimal technique of airborne infection of guinea pigs. If such methods to measure patient infectiousness and mechanical air sampling techniques for detecting viable airborne *M. tuberculosis* could be validated, it would be possible to replace this use of experimental animals in the future.

5.4 Future research

As a result of the research described in this thesis, the following further studies are in progress or are planned:

- An evaluation of low-cost, low-technology strategies appropriate for low resource settings to reduce institutional airborne TB transmission. The two strategies currently being studied are upper room germicidal ultraviolet (UV) light and the negative ionisation of air. The guinea pig air sampling facility has been completely re-designed and re-built using the experience gained through the work in this thesis. There are now three parallel animal exposure chambers, each of which may be ventilated sequentially with ward air, or with fresh air from outside. There are currently three groups of 150 guinea pigs breathing ward air on alternate days. One group is the control group, breathing untreated ward air on the first day of a two-day rotation. Simultaneously, a second group breathes ward air, but the air is treated with negative air ionisers located within that section of the animal facility. The third group of guinea pigs breathes ward air on the second day of the two-day rotation, when the upper room UV lights are switched on in the ward. The differences between the rates of TB infection in these three groups of animals will be used to evaluate the efficacy of upper room UV lights and negative ionisers in preventing airborne TB transmission. After eight months of air sampling, both interventions appear to have a considerable effect in reducing airborne TB transmission.

- Further work is planned with this facility to better define the role of upper room UV light in cleaning the air of TB. In particular studies are planned to investigate

the use of upward drawing ceiling fans to increase the efficacy of upper room UV light by augmenting air mixing between the lower and upper parts of the room. In addition, it is planned to study the optimal levels of UV intensity in a room which would allow maximal TB killing in the upper part of the room without adverse effects for room occupants. A further area of study concerning UV lights is the parallel use of dehumidifiers, since high humidity is known to significantly reduce the germicidal effectiveness of UV light.[186]

- Further work is planned with this guinea pig air sampling facility to define better the effects of negative ionisation on airborne TB transmission. In particular the optimal ion density and distribution of ionisation equipment in a room warrants further study. The effect of using UV light and negative ionisers together will also be evaluated.

- The efficacy of natural ventilation in reducing airborne TB transmission will be studied directly using the animal facility, and indirectly by studies of TB incidence in health care workers in naturally ventilated health care facilities. Modern naturally ventilated facilities will be compared with old fashioned, pre-1950 facilities with high ceilings and large windows.

- A study has already been started to evaluate natural ventilation in prisons, and the potential to improve existing natural ventilation in order to reduce the risk of airborne TB transmission in these high-risk settings. Measurements of natural ventilation using the carbon dioxide tracer gas concentration-decay technique have already been made in the health care facilities and cell blocks of six of Peru's largest prisons.

- Further work is planned to characterise the strains of *M. tuberculosis* collected in this and ongoing studies on the relative infectiousness of TB patients. It may be possible to detect differences in the strains of highly infectious TB patients compared with poorly infectious patients. Studies are being planned for further molecular typing[187] of this collection of *M. tuberculosis* strains and for the evaluation of their *in vitro* growth and cytokine induction in a macrophage infection model,[188] in collaboration with the Institute of Infectious Disease and Molecular Medicine at the University of Cape Town in South Africa.

- Plans also exist to investigate the infectivity this group of *M. tuberculosis* strains in an aerosol challenge guinea pig model. In work with patient strains of *M. tuberculosis* from South India, Balasubramanian found that strain of high virulence for guinea pigs generally had a lesion inducing efficiency (LIE) of one, that is to say that each mycobacterial colony forming unit containing droplet alighting on guinea pig lung tissue resulted in one primary focus as evidenced at autopsy six weeks later.[104] In contrast, the lower virulence strains had LIEs ranging from 1-4. Further information may be gleaned about the actual numbers of airborne mycobacteria rather than just 'quanta' present in the ward air over the 16 months of air sampling described in this thesis by defining LIEs for this collection of strains in a guinea pig aerosol challenge model.

- The data concerning the infectiousness of air from a ward containing tuberculosis patients obtained in the work described in this thesis, and additional data from the ongoing studies, will be used to help validate new airborne infection models which avoid some of the assumptions of the Wells-Riley model. New models are being developed by collaborators in the Aerobiological Research Group at the

School of Civil Engineering at Leeds University,[189] and also by another group who have developed a guinea pig air sampling facility in South Africa. Carefully collected data on tuberculosis transmission and ventilation will be important for the validation of these models, which are useful in the evaluation of the impact of environmental control measures against airborne infections.

5.5 Conclusion

The work described in this thesis provides the first evidence for the high rates of ventilation achievable in health care facilities through natural ventilation by simply opening windows and doors. A number of determinants of this natural ventilation have been elucidated, and modelling predicted a significant reduction in airborne tuberculosis transmission. In order to validate and further evaluate environmental control measures for tuberculosis transmission, a guinea pig air sampling facility has been created and optimised. This has provided new insights into the infectiousness of HIV patients with drug-sensitive and drug-resistant tuberculosis. An improved facility is currently in use evaluating alternative existing and novel environmental control strategies for the prevention of airborne tuberculosis transmission suitable for low resource settings.

CHAPTER 6

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