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RAPID IDENTIFICATION OF SELECTED PATHOGENS IN BIOTHREAT PREPAREDNESS

Markos Mölsä

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Rapid Identification of Selected Pathogens in Biothreat Preparedness

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ACADEMIC DISSERTATION

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Markos Mölsä*: Rapid Identification of Selected Pathogens in Biothreat Preparedness*

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To my family

CONTENTS

ABSTRACT

Rapid and accurate detection and diagnosis of infectious agents is crucial in preparedness for diseases and biothreats. Due to a lack of rapid diagnostic capabilities, diseases and outbreaks may remain undetected. Currently available point-of-care tests often lack the sensitivity to directly detect pathogens in samples, thus there is a need for quick and robust solutions for identification of pathogens in order to mount appropriate responses. The spread of infectious diseases is a global challenge and outbreaks and epidemics place great strains to healthcare and economy. Several of the pathogens causing these diseases are not only major public health issues but pose also potential biothreats. This thesis describes the performance of field-capable gene amplification methods in the detection of three bacterial pathogens (*Francisella tularensis*, *Bacillus anthracis*, and *Yersinia pestis*) and a viral pathogen causing respiratory infections (influenza A virus). The methods include on-site nucleic-acid extraction and rapid real-time PCR amplification of selected gene regions in these pathogens occurring in animal tissue and human nasopharyngeal samples. Results confirm that currently-available portable thermocyclers can generate highly accurate diagnostic results in field. Furthermore, genetic characterization of a common respiratory pathogen, adenovirus is presented by investigating adenoviruses circulating in Finnish garrisons with molecular sequence analysis. Genetic characterization of a pathogen is also an important tool in investigations of alleged use of biological weapons. The presented methodology and workflow serves as an effective tool for decision makers in biothreat preparedness and in case of deliberate spread of pathogens.

TIIVISTELMÄ

Biologisella uhalla tarkoitetaan mikrobin tai muun biologisen materiaalin, kuten toksiinin aiheuttamaa joukkosairastumista tai sen vaaraa, silloin kun tauti ei tartuntavaaransa vuoksi ole hoidettavissa normaalitoimin. Maailmanlaajuisiin biologisiin uhkiin on varauduttava riippumatta siitä, ovatko ne luonnollisia epidemioita tai onko taustalla taudin tahallinen levittäminen, kuten bioterrorismi tai biologisen aseen käyttö.

Biologisia uhkia voidaan vähentää tehokkaimmin ehkäisemällä epidemioiden syntyä paikallisesti. Biouhkamikrobit (esim. pernaruttobakteeri tai ebolavirus) aiheuttavat edelleen luonnollisia epidemioita. Kasvanut maailmanlaajuinen liikkuvuus on mahdollistanut taudinaiheuttajien nopean leviämisen ja lisännyt laajojen epidemioiden ja pandemian riskiä.

Biolääketieteen uudet menetelmät ja tekniikat ovat entistä helpommin ja yhä useampien saatavilla. Tämä on tuonut uusia haasteita bioterrorismin ehkäisyyn ja kansainvälisen biologiset aseet kieltävän sopimuksen valvonnalle. Bioterrorismin uhkaa voidaan vähentää tiedeyhteisön valveutuneisuutta lisäämällä sekä laboratorioturvallisuutta parantamalla ja näin estää vaarallisten mikrobien joutumista vääriin käsiin. Suomessa toimii Biologisten uhkien osaamiskeskus, joka on Terveyden- ja hyvinvoinnin laitoksen ja Puolustusvoimien logistiikkalaitokseen kuuluvan Sotilaslääketieteen keskuksen yhteinen asiantuntijaorganisaatio. Sen päätehtäviin kuuluu biologisiin uhkiin varautuminen.

Biologisiin uhkiin varautumisessa taudinaiheuttajamikrobin nopea tunnistaminen on tärkeää, sillä se mahdollistaa vastatoimien ripeän suunnittelun ja toteutuksen. Perinteiset menetelmät taudinaiheuttajien tunnistamiseksi (esim. mikrobien viljely) ovat usein hitaita tai epäherkkiä, joten molekyylibiologiset testit, kuten reaaliaikaiset geenimonistusmenetelmät, ovat saavuttaneet keskeisen aseman taudinaiheuttajien diagnostiikassa. Luotettavat, kenttäkelpoiset ja helppokäyttöiset tunnistusmenetelmät tehostavat operatiivista toimintakykyä biouhkatilanteissa. Samaa teknologiaa voidaan hyödyntää myös normaaliolojen diagnostiikassa.

Tässä väitöskirjatyössä tutkittiin kenttäkäyttöön soveltuvien kannettavien reaaliaikaisten geenimonistusteknologian käytettävyyttä ja kykyä tunnistaa luotettavasti biouhkabakteereita sekä hengitystieviruksia kenttäolosuhteissa. Työssä käytettiin kolmen biouhkabakteerin (*Francisella tularensis* (jänisruttobakteeri), *Bacillus anthracis* (pernaruttobakteeri) ja *Yersinia pestis* (ruttobakteeri)) ja influenssa A -viruksen tunnistavia menetelmiä. Työhön sisältyi lisäksi kenttäolosuhteisiin soveltuvien näytteiden esikäsittelymenetelmien tutki-

minen. Väitöskirjatyössä selvitettiin myös geenisekvensaation avulla yleisimmät suomalaisissa varuskunnissa kiertävät ja hengitystieinfektioita aiheuttavat adenoviruksen alatyypit. Geenisekvensointi on tärkeä työkalu epäillyn tahallisen levityksen tutkinnassa. Sen avulla saadaan tarkkaa tietoa taudinaiheuttajasta ja sen alkuperästä.

Tulokset vahvistivat, että nykyaikaisella kenttäkelpoisella geenimonistusteknologialla ja kenttäkelpoisilla näytteen esikäsittelymenetelmillä saadaan nopeita ja luotettavia tuloksia kenttäolosuhteissa. Tällaista teknologiaa yhdistettynä geenisekvensointiin voidaan käyttää työkaluna infektioepidemioiden selvitystyössä tai tutkittaessa epäiltyä taudinaiheuttajien tahallista levitystä. Väitöskirjatyön esittämiä menetelmiä voidaan käyttää kaikissa Puolustusvoimien päätehtävissä: maanpuolustuksessa, viranomaisyhteistyössä sekä kansainvälisissä tehtävissä.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals.

I **Markos Mölsä, Laura Kalin-Mänttäri, Elina Tonteri, Heidi Hemmilä, Simo Nikkari (2016).** Comparison of four commercial DNA extraction kits for the recovery of *Bacillus spp.* spore DNA from spiked powder samples. J Microbiol Methods. 2016 Sep;128:69-73.

II **Markos Mölsä, Heidi Hemmilä, Anna Katz, Jukka Niemimaa, Kristian M. Forbes, Otso Huitu, Peter Stuart, Heikki Henttonen, Simo Nikkari (2015).** Monitoring biothreat agents (*Francisella tularensis, Bacillus anthracis and Yersinia pestis*) with a portable real time PCR instrument. J Microbiol Methods. 2015 Aug;115:89-93.

III **Markos Mölsä, Katja Koskela, Esa Rönkkö, Niina Ikonen, Thedi Ziegler, Simo Nikkari (2012).** Detection of influenza A viruses with a portable real-time PCR instrument. J Virol Methods. 2012 May;181(2):188-91

IV **Markos Mölsä, Heidi Hemmilä, Esa Rönkkö, Maria Virkki, Simo Nikkari, Thedi Ziegler (2015).** Molecular Characterization of Adenoviruses among Finnish Military Conscripts. J Med Virol. 2016 Apr;88(4):571-7.

ABBREVIATIONS

BSL-3	biosafety level 3
BWA	Biological warfare agent
BWC	Biological Weapon Convention
BML	Bundeswehr Medical Mobile Laboratory
CBRN	Chemical Biological Radiological Nuclear
CDC	Centers for Disease Control and Prevention
cDNA	complementary DNA
CFU	colony forming units
DNA	deoxyribonucleic acid
EMlab	European Mobile Laboratory
FH	field hygiene
FRI	febrile respiratory illness
GHSA	Global Health Security Agenda
IPC	internal positive control
MSC	microbiological safety cabinet
MERS	Middle East Respiratory Syndrome
NATO	North Atlantic Treaty Organization
NGS	next generation sequencing
NTC	non-template control
OIE	Office international des épizooties, World Organization for Animal Health
PCR	polymerase chain reaction
PFU	plaque-forming unit
POC	point-of-care
PPE	personal protective equipment
Real-time PCR	real-time polymerase chain reaction
RNA	ribonucleic acid
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
SARS	Severe acute respiratory syndrome
SIBA®	Strand Invasion Based Amplification
SIBCRA	sampling and identification of biological, chemical and radiological agents
THL	National Institute for Health and Welfare
UNG	uracil-N-glycosylase
UNOG	United Nations Office at Geneva
UNSGM	United Nations Secretary General Mechanism

URTI	upper respiratory infections
WHO	The World Health Organization
WMD	weapons of mass destruction
WGS	whole genome sequencing

1. INTRODUCTION

Biological threats arise from infectious agents and toxins capable of causing disease or epidemics in human or animal populations that require extraordinary response measures. They may emerge or re-emerge directly from natural wildlife or domestic animal sources, they may be transmitted via contaminated food and water, or they may be released deliberately as modes of bioterrorism (OIE World Organization for animal health, 2016). The spread of novel pathogens is facilitated by international travel, human migration, animal trade, natural disasters and climate change. These may lead to the unintentional introduction of pathogens and increase the global risk of infectious disease epidemics or pandemics. There is also the possibility of a deliberate release of pathogens or toxins as acts of terrorism.

Rapid identification is essential for appropriate medical care, containment and control in the event of a release or natural outbreak of a contagious pathogen. Pathogens are usually identified by dedicated diagnostic laboratories and the methods typically require skilled staff, highly specific protocols and sophisticated equipment. Transporting samples to the laboratory risks a secondary release of the pathogen should an accident take place in transit, and appropriate counter-measures and medical action must wait for a confirmed diagnosis before being applied. Currently available rapid point-of-care tests often lack the analytical sensitivity to detect pathogens directly and reliably from clinical or environmental samples (Irenge and Gala, 2012, Zasada et al., 2015). As such, there is a great need for a portable, rapid and robust diagnostic tool for use at the release site wherever it may be (Irenge and Gala, 2012).

Some pathogens circulating in nature sporadically cause human diseases that can lead to epidemics, particularly in developing countries. Although naturally-occurring, these pathogens are classified as potential bioterrorism agents belonging to the highest biothreat category. These pathogens can be weaponized for deliberate use and mass destruction. Deliberate use of bioweapons may be tempting for certain individuals or groups since preparing bioweapons is simpler and cheaper than chemical or nuclear devices. Recent and rapid advances in biomedicine have made the production of bioweapons easier for independent agencies and their use to effect harm or incite terror represents a growing concern around the world (OIE World Organization for animal health, 2016).

Other pathogens such as respiratory viruses are causing significant outbreaks of illness throughout the world, especially in crowded places such as kindergartens, schools and military garrisons (Gray et al., 1999, O´Shea MK and Wilson D, 2013).

The main purpose of this thesis was to demonstrate that modern portable thermocycler technology can generate data of a similar quality to that of dedicated laboratories with a fixed location. Field-deployable, point-of-care molecular diagnostic tools and molecular characterization of the pathogen can be used to monitor epidemics or to investigate suspected use of biological weapons.

2. REVIEW OF THE LITERATURE

2.1 Biological threats

Biological threats may emerge directly from natural wildlife sources, or they may be released deliberately as acts of bioterrorism (OIE World Organization for animal health, 2016, National Institute for Health and Welfare, 2014a).

Some biothreats cause diseases such as plague and anthrax and are classified as potential bioterrorism agents in the highest biothreat category (Centers for Disease Control and Prevention (CDC), 2016a, Ryan, 2008). These pathogens can be turned into weapons and used to infect a target population. The use of such weapons could result in a massive loss of life and cause economic, political, and social damage from local to global scale (Ryan, 2008). Progress in genetic engineering, synthetic biology and biomedical technique has simplified the development, production, storage and use of biological weapons (Kelle, 2013, Konig et al., 2013, Moe-Behrens et al., 2013, Riedel, 2004). In the wrong hands, just one of the deadly viruses or bacteria could cause a catastrophic loss of life. The possibility of using biological agents for malevolent purposes represents a growing concern for law enforcement, governments, and public health officials around the world (OIE World Organization for animal health, 2016). In response, initiatives such as the United Nations Secretary General Mechanism (UNSGM) for investigating the alleged use of chemical, biological or toxin weapons (United Nations Office for Disarmament Affairs, 2016) have been developed.

2.2 Zoonotic diseases – a global challenge

Zoonotic diseases, or zoonoses, are infectious diseases caused by naturally-occurring pathogens that circulate within animal populations and which can be transmitted to humans under natural conditions. Zoonotic diseases are often categorized according to their route of transmission, pathogen type or infectivity among humans (Lloyd-Smith et al., 2009). Emerging infectious diseases are those that have increased recently or threaten to increase in the near future. Diseases that once were endemic and had since been eradicated or controlled but are again becoming health problems are re-emerging diseases (Dikid et al., 2013). Emerging and re-emerging diseases have caused many pandemics in human history, e.g., the Black Death in 1346–1353 by *Yersinia pestis* and Span-

ish Influenza in 1918-1920 by influenza A H1N1 (Bos et al., 2011, Morens et al., 2008, Weber and Rutala, 1999, OIE World Organization for animal health, 2016, Woolhouse and Gowtage-Sequeria, 2005).

New infectious diseases emerge because of many factors. Global processes such as climate change, rapid intercontinental travel, mass human migration, animal production and foreign trade with countries having high prevalence of pathogens and their vectors have all increased the risk of novel biological threats (Courtney et al., 2014, Lindahl and Grace, 2015). Urbanization implies a greater concentration and connectedness of people and may increase the speed at which new infections can emerge and spread. Furthermore, income growth is associated with rising animal protein consumption in developing countries, which increases the conversion of wild lands to livestock production (Wu et al., 2016).

Several human infectious diseases arose after the introduction of new agricultural techniques (Wolfe et al., 2007). Nowadays, food-borne diseases are recognized as a high priority as there are many stages at which food safety can be compromised (Cohen, 2000). Land-use changes (e.g., agricultural encroachment, deforestation, road construction, etc.) are drivers of infectious disease emergence and outbreak (Patz et al., 2004). Land modification and deforestation have a direct impact on vegetation, an indirect impact on host-vector dynamics, and increase human contact with wild animals and their zoonotic pathogens. An additional and often overlooked aspect of natural areas is their rich microbial diversity (Jones et al., 2008). Anti-microbial drug resistance has enormous significance for global health and is a serious problem (Cohen, 2000, Jones et al., 2008) that might only be solved by the identification and development of biochemicals developed from existing microbes. Drug-resistant microbes represent a significant issue related to biothreat agents and bioterrorism (Zakowska et al., 2015).

More than 75 % of human diseases are zoonotic and have links to wildlife and domestic animals (Taylor et al., 2001). Zoonotic infection of a local human population can occur in many ways. Hunting, butchery and consumption of wild animals have led to the zoonotic transmission of several diseases (Hahn et al., 2000). Most of these (e.g., severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS), pandemic influenza, and ebola) are caused by animal pathogens transmitted from wildlife reservoirs to humans, often through an intermediate amplification host which are the source of infection for humans (Carroll et al., 2015, Gardy et al., 2015, Guan et al., 2003, Haydon et al., 2002, Jones et al., 2008, Lagace-Wiens et al., 2010, Lebarbenchon et al., 2013, Rewar et al., 2015, Rouquet et al., 2005). Zoonoses are also transmitted by house pets (Damborg et al., 2015). In recent decades the world has faced new disease

threats arising from the increasing ease and volume of air travel creating a single "global" community (Figure 1).

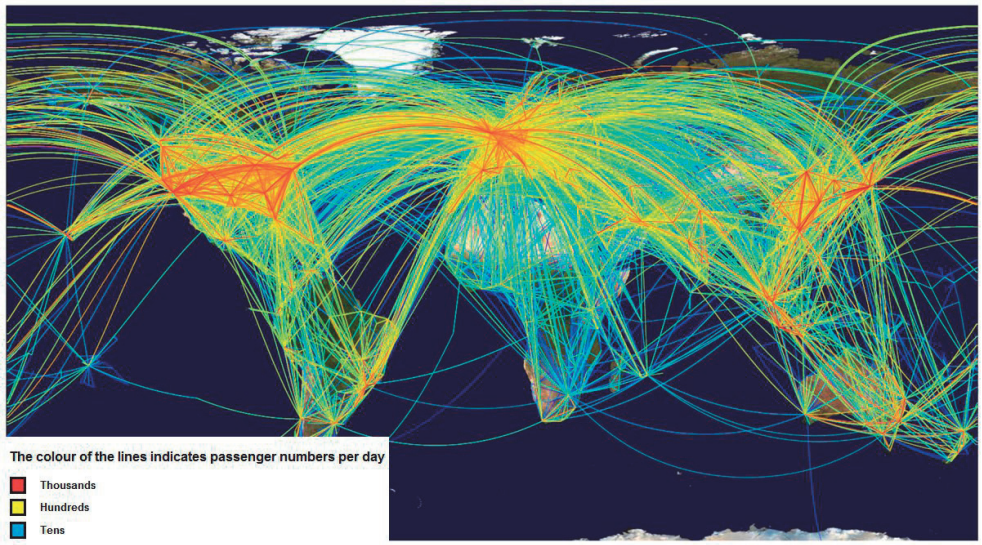


Figure 1. Global aviation network of today. Lines show connections between airports, and the color of the lines indicates passenger numbers per day. Modified from (Kilpatrick and Randolph, 2012).

Zoonoses tend to affect developing countries and rural areas with poor access to health services where malnutrition can enhance susceptibility to infection (Cohen, 2000, Coker et al., 2011). Detection and control of diseases in situations of conflict and war are a major challenge. Conflicts result in a loss of adequate surveillance and response as well as a collapse of infrastructure and healthcare (Gayer et al., 2007). However, threats are not only limited to low-income countries or conflict zones due to expanded global trade and air travel (Karesh et al., 2012). The rapid spread of global diseases such as SARS and influenza demonstrates the speed with which human epidemics can emerge (Coker et al., 2011).

The spread of zoonotic diseases is a global challenge as they represent a significant cause of illness and death each year (Cohen, 2000, Karesh et al., 2012). They place a great strain on healthcare and are the reason for huge economic losses in some countries (Jones et al., 2008, Karesh et al., 2005). Respiratory infections, HIV/AIDS and diarrheal diseases account for the most deaths attributable to infectious disease (Morens et al., 2004).

Effective prevention, enhanced biosafety and biosecurity measures, in concert with efficient detection technology are required to reduce the spread of infectious diseases. To develop and deploy rapid diagnostic tests and to train people in using them will improve response to these infections and their outbreaks in an early phase when control measures are most effective in limiting the spread to the human population (Centers for Disease Control and Prevention (CDC), 2016b).

2.3 Selected high-priority agents

The US CDC classifies pathogens according to their risk of infection and scale of threat. Category A pathogens are high-priority agents that pose a risk to national or global security (Table 1). These agents can be easily spread or transmitted from person to person, are extremely virulent and result in high mortality rate, thus have the potential for a major public health impact. Release of such agents may result in panic and social disruption and requires special attention and action in order to attain public health preparedness (Katz and Zilinskas, 2011d). Category B pathogens are the second highest priority as they are moderately easy to spread and they result in low or moderate morbidity and mortality rates (Table 1). Category C pathogens could be engineered for deliberate use and they are more easily available than A and B category agents but also have the potential to create a major health impact (Table 1) (Katz and Zilinskas, 2011d).

Table 1. List of risk-category A, B, and C pathogens and the diseases they cause (Katz and Zilinskas, 2011d).

List of Category A-C agents		
	Agent	**Disease**
Category A	*Francisella tularensis*	Tularemia
	Bacillus anthracis	Anthrax
	Yersinia pestis	Plague
	Clostridium botulinum toxin	Botulism
	Variola major	Smallpox
	Arenaviruses, Bunyaviruses, Flaviviruses, Filoviruses	Viral hemorrhagic fevers
Category B	*Brucella species*	Brucellosis
	Clostridium perfringens (Epsilon toxin)	Foodborne illness
	Salmonella spp., *Escherichia coli 0157:H7, Shigella* spp. (foodborne pathogens)	
	Burkholderia mallei	Glanders
	Burkholderia pseudomallei	Melioidosis
	Chlamydia psittaci	Psittacosis
	Coxiella burnetii	Q fever
	Ricinus communis, (ricin toxin), castor beans	
	Staphylococcus aureus, (staphylococcal Enterotoxin B), SEB	
	Rickettsia prowazekii	Typhus fever
	Alphaviruses	Viral encephalitis
	Vibrio cholerae	Cholera
	Cryptosporidium parvum	Cryptosporidiosis (parasitic disease)
	Influenza A virus	Influenza
Category C	Tickborne hemorrhagic fever viruses	
	Tickborne encephalitis viruses	
	Yellow fever virus	Yellow fever
	Multidrug resistant tuberculosis	Tuberculosis
	Other *Rickettsias*	
	Rabies virus (*Lyssavirus* spp.)	Rabies
	Severe acute respiratory syndrome-associated coronavirus (SARS-CoV)	

CDC lists three bacterial species as category A biological threat agents: *Francisella tularensis, Bacillus anthracis* and *Yersinia pestis*. These bacterial agents are zoonotic and highly pathogenic and virulent, potentially causing high case fatality and thus pose a major risk to public health (Anderson and Bokor, 2012). The risks these bacteria pose are amplified by their ability to be transmitted through aerosols (Katz and Zilinskas, New Jersey:

John Wiley & Sons, Inc; 2011.), and an incubation period of up to several weeks following human exposure (Anderson and Bokor, 2012, Dennis et al., 2001, Koskela and Salminen, 1985) which makes determining the source of infection more difficult.

These three pathogens can present as invisible, odorless and tasteless aerosols and their release can be difficult to detect initially in a population that is unaware of its exposure. Related to this is the issue of psychological damage and stress suffered by a public facing a constant threat of biological agents (Katz and Zilinskas, 2011e). Rapid identification and accurate characterization of these agents is therefore essential for the swift application of appropriate counter-measures as well as primary care and control protocols. Moreover, timely post-exposure antibiotic treatment, enabled by rapid detection and identification, can protect against infections caused by these agents (Irenge and Gala, 2012, Ivnitski et al., 2003).

2.3.1 *Francisella tularensis*

Francisella tularensis is the causal agent of the zoonotic disease tularemia. In addition to the mode of transmission, severity of illness depends on bacterial subtype. The clinically most relevant subtypes are *F. tularensis tularensis* (type A) and *F. tularensis holarctica* (type B). *F. tularensis tularensis* is highly pathogenic for humans and is the main causal agent of tularemia in North America, whereas *F. tularensis holarctica* is less virulent and widely distributed in many animal species in Eurasia (Tarnvik et al., 2004). Tularemia presents a wide variety of primary clinical symptoms in humans, which depend on the route of infection. The infectious dose of *F. tularensis* is low. Infection in humans can occur after exposure to as little as 10–50 colony-forming units (CFUs) and the incubation period for tularemia can range from 1 to 14 days (Dennis et al., 2001, Koskela and Salminen, 1985). The bacteria can be transmitted by insect bites, contaminated water, food and aerosols (Foley and Nieto, 2010) and remain infectious over long periods of time in cool and humid environments (Whitehouse and Hottel, 2007). In Fennoscandia, the disease is believed to be mainly transmitted via mosquitoes. However, the ecology of tularemia is not clearly understood and the natural reservoir of the bacterium is not yet established, although rodents are suspected to be involved. In support of a rodent reservoir, tularemia outbreaks are associated with poor hygienic conditions, especially in war and post-war situations when rodent densities are typically high (Grunow et al., 2012). Several tularemia outbreaks have been described (Rossow et al., 2014), including cases in the Soviet Union during World War II (Sjostedt, 2007) and in Scandinavian and other European countries, (Christenson, 1984, Dahlstrand, Sverker., Ringertz, Olof., Zetterberg, Bo., 1971, Splettstoesser et al., 2009). In Finland, tularemia (*F. tularensis holarctica*) is endemic and seasonal cases are seen in the late summer and early fall (Ros-

sow et al., 2014, Tarnvik et al., 2004). During 1995–2016, the National Infectious Diseases Register received 5832 notifications of laboratory confirmed tularemia cases (Figure 2).

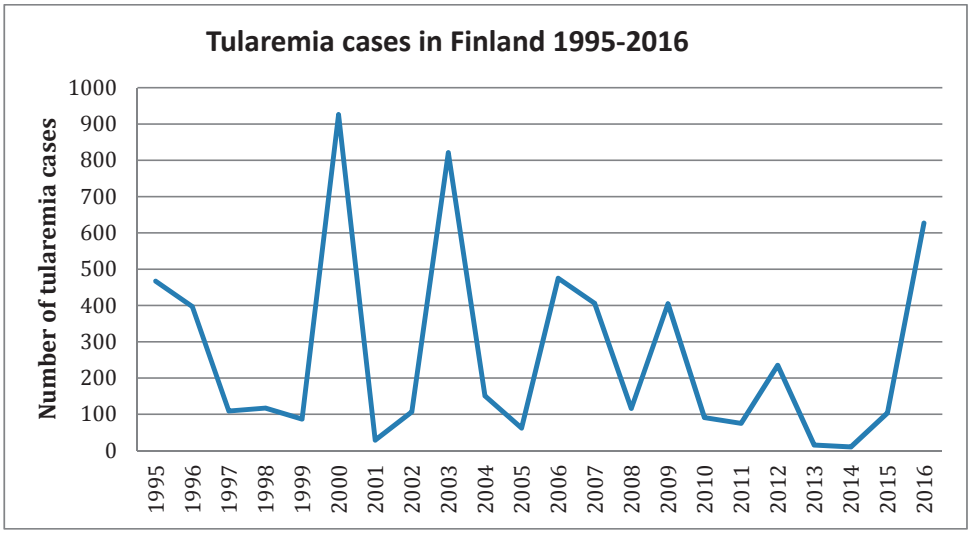


Figure 2. Since 1995, nearly 6000 cases of tularemia have been reported. Rodents act as amplification hosts of *F. tularensis*, and high rodent densities predict tularemia cases in humans in the following year (Rossow, 2015).

2.3.2 *Bacillus anthracis*

B. anthracis is an endospore-forming bacteria and the causal agent of anthrax. The bacterium forms resistant spores that enable the organism to survive long periods in harsh conditions outside of an animal host (Williams et al., 2013). Three forms of anthrax are known. In the cutaneous form, spores of *B. anthracis* pass through minor skin breaks, resulting in the formation of dermal ulcers. Over 95 % of anthrax cases are of cutaneous form and result from direct contact with spores or live bacteria (Anderson and Bokor, 2012). The second form is gastro-intestinal, which occurs most commonly after ingestion of poorly-cooked meat contaminated with spores. The third form is inhalation anthrax, caused by breathing in spores. Gastrointestinal and inhalation anthrax have high case fatality rates when not treated. The infective dose of *B. anthracis* is reported to be approximately 8000–10000 spores (Cowcher et al., 2013, Katz and Zilinskas, 2011c). The incubation period for inhalational anthrax is usually less than a week but in some rare cases much longer. Person-to-person transmission of anthrax has not been reported (Anderson and Bokor, 2012). The disease occurs naturally in many parts of the world with the highest incidence in sub-Saharan Africa and central Asia (Katz

and Zilinskas, 2011a). Furthermore, both accidental and deliberate releases of *B. anthracis* have been reported (Dixon et al., 1999, Jernigan et al., 2002, U.S Department of Defence, 2015, Sternbach, 2003).

2.3.3 *Yersinia pestis*

Yersinia pestis is the causal agent of the systemic invasive infectious disease, plague. The bacterium is primarily a rodent pathogen, usually transmitted to humans by the bite of an infected flea, but can also be transmitted by air (saliva droplets) during plague pandemics and epidemics (Parkhill et al., 2001, Raoult et al., 2013, World Health Organization, 6 September 2015). Pneumonic plague may also spread from person-to-person (Butler, 2009, Raoult et al., 2013). The estimated infective dose of *Y. pestis* is approximately 1000 bacterial cells. The infective dose of *Y. pestis* is therefore less than for *B. anthracis*, but more than *F. tularensis* (Katz and Zilinskas, 2011c). The incubation period for airborne plague can range from 1 to 10 days (Anderson and Bokor, 2012), (World Health Organization, 2016). Most symptoms will subside with antibiotic treatment. Plague has caused several major epidemics in Europe and Asia over the last 2000 years, and in the 14th century it killed more than one-third of the population of Europe within a few years (Katz and Zilinskas, 2011c). Furthermore, allegations of the use of *Y. pestis* during World War II have been presented (Frischknecht, 2003). Today, up to 3000 cases of plague are reported annually to the World Health Organization (World Health Organization, 2005).

2.4 Common pathogens causing respiratory infections in Finnish garrisons

Respiratory tract infections cause morbidity and mortality worldwide (Zumla et al., 2014). Upper respiratory tract infections (URTI) are the most common infectious diseases among persons of all age groups and they are mostly caused by viruses. Occasionally these viruses reach the lower respiratory tract, causing bronchitis, bronchiolitis and pneumonia, which can be severe, even life-threatening (Jamison DT, Breman JG, Measham AR, et al., 2006). Respiratory viruses are easily transmitted in high population densities, such as in day-care centers, schools, and military garrisons. Overcrowding, physical and mental stress and frequent traveling may predispose an individual to respiratory tract pathogens (Gray et al., 1999, O´Shea MK and Wilson D, 2013). Respiratory infections have been recognized for a long time as common illnesses among military recruits during their service (Sanchez et al., 2001, Top, 1975). Adenoviruses, influenza A and B viruses, *Streptococcus pneumoniae* and *Streptococcus pyogenes* have been recog-

nized as the primary causal agents in acute respiratory infections (ARI) of army personnel (Gray et al., 1999, Wang et al., 2010).

Respiratory viruses are the source of significant outbreaks of illness among military conscripts in Finland each year, commonly during late winter and early spring (Hulkko et al., 2010). Finnish garrisons actively participate in the sentinel surveillance of viral respiratory infections coordinated by the National Institute for Health and Welfare (THL). This surveillance provides detailed information on the circulation of various respiratory viruses in the Finnish population as well as on the characteristics of these viruses. Outbreaks of influenza A and adenoviruses are registered every year. Other common viruses causing respiratory infections are presented in the sentinel surveillance material (Figure 3) (Hulkko et al., 2010). Kauppila et. al (2014) monitored respiratory infections in military recruits in eastern Finland in 2004–2005. PCR results were positive for the presence of influenza virus types A, B, and C, adenovirus, parainfluenzavirus types 1, 2 and 3, respiratory syncytial virus (RSV), human metapneumovirus (hMPV), rhinovirus, enterovirus, *Chlamydia pneumoniae,* and *Mycoplasma pneumoniae* (Kauppila et al., 2014). Furthermore, microbiological swabs taken during acute rhinosinusitis in a cohort of 50 Finnish military recruits included *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Neisseria meningitis* (Autio et al., 2015). Particularly in garrisons, epidemics caused by respiratory infections are often characterized by a sudden onset, and the rapid identification of the pathogen may help in planning appropriate countermeasures and treatment strategies. Some of the common respiratory pathogens are also related to severe epidemics or pandemics (i.e., influenza A and adenoviruses) and have been detected in military personnel (Byerly, 2010, Hoke and Snyder, 2013, Morens et al., 2010).

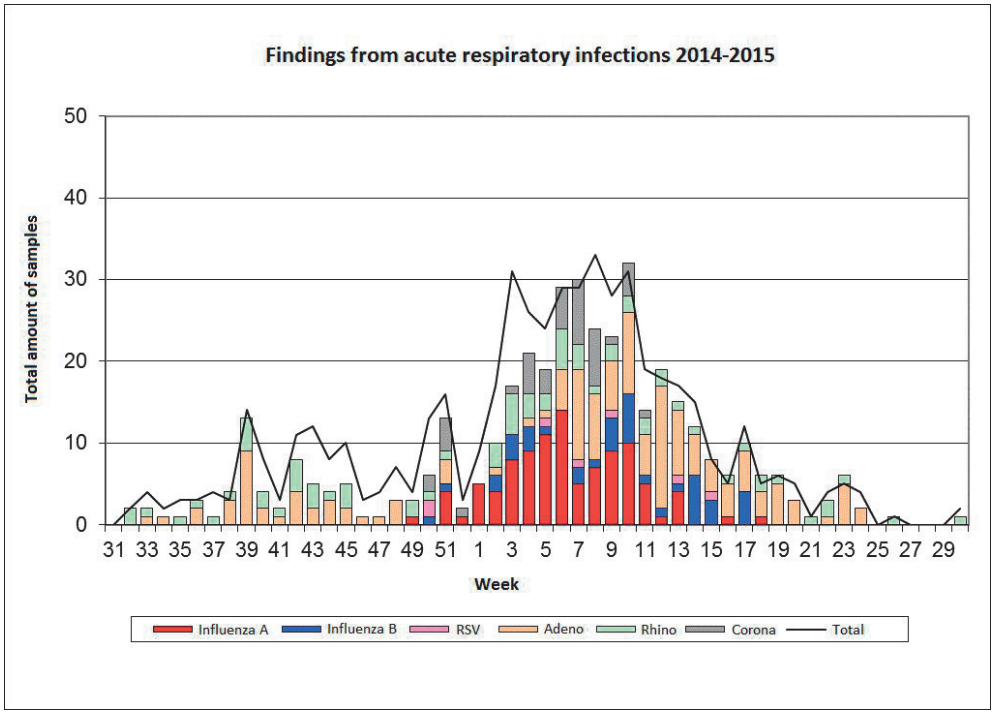


Figure 3. Findings from acute respiratory infection surveillance during 2014-2015 in Finnish garrisons. Modified from (National Institute for Health and Welfare).

2.4.1 Influenza A viruses

Influenza viruses are a major cause of human acute respiratory disease globally (Katz and Zilinskas, 2011b). In Finland, annual epidemics caused by influenza A virus are recorded with morbidity and mortality. Therefore, risk groups are vaccinated free of charge through a national vaccination program. In the Finnish Defense Forces seasonal influenza epidemics are seen annually, particularly among military recruits undergoing basic training during the winter months (Hulkko et al., 2010). An influenza vaccine has been offered to all conscripts entering service since 2012.

Influenza viruses are members of the orthomyxovirus family and there are four types known as A, B, C and D. Influenza A and B viruses are both responsible for the annual epidemics. Influenza C viruses cause low-level sporadic disease with limited outbreaks and occur almost exclusively in humans (Cox and Subbarao, 2000, Taubenberger and Morens, 2010). Influenza D virus is a novel pathogen with bovine as its primary host (Sreenivasan et al., 2015). Influenza A can sporadically cause pandemics (Cox and Subbarao, 2000).

Influenza ecology is complicated because of the presence of avian and mammalian viral reservoirs. Several bird species serve as large reservoirs for influenza A viruses (Cox and Subbarao, 2000, LaForce et al., 1994). Influenza A viruses can occasionally jump from one host species to another and subsequently diversify into one or more new lineages within the new host (Taubenberger and Morens, 2010). Avian influenza viruses can sometimes infect mammals, and pigs are often infected with both avian and mammalian strains (Cox and Subbarao, 2000). Therefore, pigs may serve as a reservoir for the mixing and re-assortment of mammalian and avian strains, potentially resulting in antigenic shift. This potential for the pig to function as a viral mixing vessel has been used partly to explain why China and Southeast Asia have been a source of past epidemic and pandemic influenza viruses. In these regions, humans, domestic fowl and pigs occur at a density not seen anywhere else on Earth (Cox and Subbarao, 2000), (LaForce et al., 1994). Amplification of influenza viruses during epidemics and pandemics can potentially lead to new and possibly more virulent viruses, or to viruses resistant to antiviral drugs (Lagace-Wiens et al., 2010).

In the northern hemisphere, seasonal influenza typically circulates from November to March (Cox and Subbarao, 2000, Monto, 2008). The primary modes of transmission are saliva-droplet aerosols and direct contact with the virus on contaminated surfaces and foods. Increased crowding during the colder months (e.g., in schools, kindergartens and military barracks) is a contributing factor to epidemics (Cox and Subbarao, 2000, LaForce et al., 1994, Monto, 2008). Typical seasonal influenza infections are associated with classical symptoms, such as four to five days of fever, chills, headache, muscle pain, weakness and often upper respiratory symptoms and cough (Monto, 2008). The average annual influenza mortality in developed countries is approximately 12 in 100,000 persons (Monto, 2008).

Followed by the appearance of a new influenza A subtype in humans, three major pandemics have occurred during the 20th century; the 1918–1919 pandemic (H1N1), the 1957–1958 pandemic (H2N2) and the 1968 pandemic (H3N2) causing many deaths as well as a considerable economic impact (Cox and Subbarao, 2000, Kilbourne, 2006, Morens et al., 2010). In spring 2009, a novel influenza A virus emerged in North America and rapidly spread around the globe (Gardy et al., 2015).

In June 2009, the World Health Organization declared the onset of the first influenza pandemic in over 40 years (CDC, 2010, Lagace-Wiens et al., 2010, Morens et al., 2010). Epidemiological studies have shown that elderly individuals had protective antibodies against this novel virus (Ikonen et al., 2010). In addition, (H1N1)pdm09 viruses resistant to antiviral neuraminidase inhibitors have been detected only occasionally (Laga-

ce-Wiens et al., 2010). Fortunately, the 2009 A/H1N1 pandemic virus was not as pathogenic as, for example, H5N1 (Peiris et al., 2004). Nevertheless, the World Bank estimated the viral pandemic cost the global economy close to three trillion US dollars (Lagace-Wiens et al., 2010).

Advances in healthcare and technology have had a major impact on influenza mortality figures. Increased public awareness, preventive strategies, early diagnosis and treatment have improved dramatically. However, as a large portion of the global population does not have access to the same level of prevention and medical care as developed countries, challenges remain in medical capacity and resource availability (Morens et al., 2010).

2.4.2 Adenoviruses

Adenoviruses are a common cause of acute respiratory disease globally. For many years, acute respiratory illnesses associated with adenoviruses have been reported among adults and military populations (Gray et al., 2000). The first human adenovirus was isolated in 1954 in the United States from military recruits with febrile respiratory illness (FRI) (Hilleman and Werner, 1954). Epidemics caused by adenoviruses can exhibit high levels of morbidity (Potter et al., 2012), and they can cause a wide range of clinical manifestations, ranging from mild to severe infections. The most common clinical symptoms of adenovirus infections are similar to the common cold and an upper respiratory infection often accompanied by fever, and occasionally developing into bronchitis and pneumonia (Kunz and Ottolini, 2010). Some adenoviruses are related to follicular conjunctivitis or pharyngoconjunctival fever and highly contagious keratoconjunctivitis and tonsillitis (German et al., 2008, Lenaerts et al., 2008, Ylikoski and Karjalainen, 1989). Among immunocompetent individuals, adenovirus infections are generally mild but sometimes, particularly in immunocompromised patients, adenovirus infections can cause severe disease (Tebruegge and Curtis, 2012). Currently, over 60 human adenovirus serotypes are known and are divided into subgroups A–G (Ghebremedhin, 2014, Robinson et al., 2013). Among adults, adenovirus subgroup B (serotypes 3, 7 and 21), subgroup C (serotypes 1, 2 and 5) and subgroup E (serotype 4) are common causes of respiratory tract infections and have been associated with outbreaks (Brosch et al., 2009, Kajon et al., 2015, Lu et al., 2013).

A vaccine against certain adenovirus serotypes was first introduced in 1971 but the manufacturer then ceased production in 1996. In 2011, the US Food and Drug Administration approved a program to resume vaccinating recruits with an oral vaccine against adenovirus types 4 and 7, resulting in a dramatic decrease of adenovirus-associated res-

piratory disease in army training centers (Hoke and Snyder, 2013, Potter et al., 2012, Radin et al., 2014). Kajon et al. (2015) reported the genomic characterization of adenovirus types from unvaccinated US military recruits presenting FRI symptoms during a 15-year period (1996–2011). Additional strains isolated from civilians were characterized for comparison. Results suggested that adenovirus type 4 was the dominant causal agent of FRI among adults. Furthermore, adenovirus types 7, 3, 14 and 21 were well represented (Kajon et al., 2015, Kajon et al., 2010). In the 1960s, Mäntyjärvi et al., (1966) recognized by serological methods that mainly adenovirus serotypes 3, 4 and 14 were circulating in Finnish servicemen (Mantyjarvi, 1966). Overall, the Finnish Defense Forces has had little information on the adenovirus serotypes circulating in Finland.

2.5 Biological Warfare Agents (BWA)

Infectious agents were used in warfare as early as 600 BC. The use of filth, cadavers, animal carcasses and contagion was noticed to weaken the enemy. Also, polluting a water supply was a commonly used tactic in siege warfare (Barras and Greub, 2014, Riedel, 2004, Wagar, 2016). The use of biological weapons became more sophisticated as microbiology made the isolation and production of specific pathogens possible and affordable (Riedel, 2004). These and other contamination strategies were used in many wars. Allegations of the use of cattle inoculated with disease-producing bacteria were reported during World War I (Riedel, 2004, Wagar, 2016) (Table 2). During and after World War II, several countries had offensive biological warfare research programs (Table 2) (Carus, 2015, Riedel, 2004). These programs focused on pathogens that could be easily spread and which were very infective with a significant mortality rate. Nowadays, these pathogens are listed in risk categories A–C by the CDC (CDC, 2015) (Table 1). Furthermore, several assassination attempts and terrorist attacks as well as allegations related to biological warfare research have been reported (D'Amelio et al., 2015, Riedel, 2004). The exact use of biological weapons in history remains difficult to determine because microbiological and epidemiological data are lacking, and the weight of political propaganda and issues with military secrecy will always obscure the real picture (Barras and Greub, 2014).

Table 2. Examples of biological warfare use during World War I and II.

World War I	German and French agents used glanders (*Pseudomonas pseudomallei*) and anthrax (*Bacillus anthracis*)
World War II	Japan used plague (*Yersinia pestis*), anthrax (*Bacillus anthracis*), and other pathogens, and several other countries experimented with and developed biological weapons programs

2.6 The Biological Weapon Convention (BWC)

In response to the use of chemical warfare during World War I, efforts were made to limit the proliferation and use of biological and chemical weapons. On June 17, 1925, the "Protocol for the Prohibition of the Use in War of Asphyxiating, Poisonous or Other Gases and of Bacteriological Methods of Warfare," commonly called the Geneva Protocol of 1925, was signed by 108 nations. The Geneva Protocol did not address verification or compliance, making it a less meaningful document. Thus, during the late 1960s, concerns were raised regarding epidemiologic risks and the lack of control measures for biological weapons (Riedel, 2004).

The Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction, commonly known as the Biological Weapons Convention (BWC), opened for signature in 1972 and became active in 1975 (The United Nations Office at Geneva (UNOG)). Finland ratified the convention in 1974. The BWC was the first multilateral disarmament treaty banning an entire category of weapons, and is a key element in the international community's efforts to address the proliferation of weapons of mass destruction. It further required the parties to the BWC to destroy stockpiles, BWA delivery systems, and production equipment within nine months of ratifying the treaty (Riedel, 2004, The United Nations Office at Geneva (UNOG)). The BWC prohibits the development, production, stockpiling, acquisition or retention of microbial or other biological agents, or toxins whatever their origin or method of production, of types that have no justification for prophylactic, protective or other peaceful purposes, also weapons, equipment or means of delivery designed to use such agents or toxins for hostile purposes or in armed conflict (The United Nations Office at Geneva (UNOG)). Under the convention, the development of delivery systems and the transfer of biological warfare tech-

nology or expertise to other countries are also prohibited (The United Nations Office at Geneva (UNOG)).

At present, BWC has 175 state parties and nine signatories (Table 3) while there are 13 states which have neither signed nor ratified the convention (Table 4) (The United Nations Office at Geneva (UNOG), 2015). The Seventh Review Conference in 2011 agreed "that a concerted effort by states parties is needed to persuade states not party to join the Convention" (The United Nations Office at Geneva (UNOG), 2011).

Table 3. Nine states have signed but not ratified the BWC as of October 2016.

States with signatories but not ratified
Angola, Central African Republic, Egypt, Haiti, Liberia, Nepal, Somalia, Syrian Arab Republic, United Republic of Tanzania

Table 4. Thirteen States have neither signed nor ratified the BWC as of October 2016.

States neither signed nor ratified
Chad, Comoros, Djibouti, Eritrea, Guinea, Israel, Kiribati, Micronesia, Namibia, Niue, Samoa, South Sudan, Tuvalu

2.7 Biosafety and Biosecurity

Biosafety determines the principles, technologies, practices and measures to prevent the accidental release of or unintentional exposure to biological agents and toxins (Dickmann et al., 2015). A biosafety protocol determines who may handle the pathogens and where they are handled, as when working with pathogens there is always a risk to be considered and assessed. It also includes the control of biological samples and material related to samples, i.e., an inventory of samples and laboratory devices (Dybwad et al., 2013, The United Nations Office at Geneva (UNOG), 2008, Weiss et al., 2015, World Health Organization, 2004).

Biosecurity aims to prevent pathogens ending up in the wrong hands (Jernigan et al., 2002). Special microbiological practices enhance work safety and environmental protection. Pathogen risk groups require different levels of containment (Table 5) (World Health Organization, 2004). In Finland, the Ministry of Social Affairs and Health has also set a classification of biological agents for protection of laboratory personnel related to dangers caused by biological agents (Ministry of Social Affairs and Health).

Table 5. The World Health Organization (WHO) has established an agent risk group classification for laboratory use that describes four general risk groups. They correlate with but do not equate to biosafety levels (World Health Organization, 2004).

Risk group classification	
Risk group 1	No or low individual and community risk. A microorganism unlikely to cause human or animal disease.
Risk group 2	Moderate individual risk and low community risk. A pathogen that can cause human or animal disease but is unlikely to be a serious hazard.
Risk group 3	High individual risk and low community risk. A pathogen that usually causes serious human or animal disease but does not ordinarily spread from one infected individual to another.
Risk group 4	High individual and community risk. A pathogen that usually causes serious human or animal disease and can be readily transmitted from one individual to another. Effective treatment and preventive measures are not usually available.

Biosafety Level 1 (BSL-1) is suitable for work involving well-characterized agents that present minimal potential hazard to laboratory personnel and environment. BSL-1 laboratories are not necessarily of restricted access beyond that of the main building (World Health Organization, 2004). Biosafety Level 2 (BSL-2) is suitable for work involving agents that pose a moderate hazard to personnel and the environment. Laboratory personnel have specific training in handling pathogenic agents. Access to the laboratory is restricted when work is being conducted (World Health Organization, 2004). Biosafety Level 3 (BSL-3) is suitable for work with indigenous or exotic agents which may cause serious or potentially lethal disease through the inhalation route or other exposure. Laboratory personnel must receive specific training in handling pathogenic and potentially lethal agents. A BSL-3 laboratory has special engineering and design features (World Health Organization, 2004). Biosafety Level 4 (BSL-4) is required to work with dangerous and exotic agents that pose a high individual risk of aerosol-transmitted laboratory infections and life-threatening disease that is frequently fatal, for

which there are no vaccines or treatments. Laboratory personnel must have specific training in handling extremely hazardous infectious agents. BSL-4 laboratories have special engineering and design features to prevent microorganisms from being disseminated into the environment (World Health Organization, 2004).

Biosecurity refers to "protection, control and accountability measures implemented to prevent the loss, theft, misuse, diversion or intentional release of biological agents and toxins and related resources as well as unauthorized access to, retention or transfer of such material" (Dickmann et al., 2015).

Related to biosafety and biosecurity there is also a risk of dual use of biological material (Resnik, 2009). Dual-use research includes, for example, enhancing the pathogenic potential of a microorganism, engineering a pathogen to render it undetectable with known detection methods or assays, or making a pathogen resistant to vaccines or to antibiotics or antiviral drugs. Scientists have a professional and ethical responsibility to understand the concept of dual use as there is a potential risk that biological material, technologies or results from the research may end up being used for harmful purposes. Sharing information, risk assessments and response mechanisms in order to strengthen biological safety, security and preparedness can minimize the risk (Kozlovac and Schmitt, 2015, Patrone et al., 2012). Dual use should also be considered when creating new biological substances that could possibly cause serious harm, or producing new delivery systems for biological substances with aerosols, drinking water, foodstuffs, etc. (Tumpey et al., 2005). Progress in biotechnology (Konig et al., 2013, Moe-Behrens et al., 2013) has simplified the development and production of new substances (Kelle, 2013, Riedel, 2004). As an example, Tumpey et al. (2005) reconstructed the 1918 Spanish influenza pandemic virus and studied its properties in cell cultures and in mice. The research community discussed the risk of intentional spread of this deadly virus to the human population (Tumpey et al., 2005). Another example of potential risk of dual use are synthesized biological pathogens. Cello et al. (2002) presented chemical synthesis of poliovirus cDNA by preparing a generation of infectious virus in the absence of the natural template (Cello et al., 2002, Wimmer, 2006). However, this is technically demanding with the current technology.

Weapons of mass destruction (WMD) are nuclear, chemical and biological weapons. Proliferation of WMDs is a serious concern and constitutes a threat to international peace and security, and several treaties have been concluded to prevent the spread of these weapons. These treaties include the BWC and the Chemical Weapons Convention (CWC). Finland is fully committed to the implementation of these conventions (University of Helsinki - Verifin, 2009). The Finnish Biosafety and Biosecurity Network was founded in 2012, including both governmental and non-governmental parties. The

network aims to promote biosafety and biosecurity and related practices in Finnish microbiological laboratories, currently focusing on BSL-3. The main activities of the network are to provide education and to raise awareness through training and seminars on biosafety and biosecurity, biorisk management, dual use and best laboratory practices (National Institute for Health and Welfare, 2014b).

2.8 Mobile biological laboratories

The advantages of mobile laboratories have been recently recognized (Wolfel et al., 2015, Grolla et al., 2011, Inglis et al., 2008). For example, the Bundeswehr Medical Mobile Laboratory (BML) and the European Mobile Laboratory (EMLab) are able to diagnose and investigate unusual disease outbreaks or events at the site of infection or release, e.g., in the recent Ebola outbreak in West-Africa (Wolfel et al., 2015). Field laboratories such as BML and EMLab comprise inflatable tent structures containing sealed partitions in which all supplies and equipment can be maintained and operated under clean and controlled conditions (Grolla et al., 2011, Wolfel et al., 2015).
Field laboratories have been set up to support disease control efforts. The ability to place laboratory services close to an infected population can help with the early identification of the pathogen involved (Inglis et al., 2011). As such, field laboratories must be sufficiently portable, robust and able to operate independently (Inglis, 2013). Rapid and accurate identification of the causal agent(s) is critical for the effective containment of outbreaks and for providing appropriate care to those exposed (Grolla et al., 2012, Inglis, 2013, Towner et al., 2006). Biosafety in a mobile laboratory is achieved by having highly trained and experienced personnel applying optimized laboratory protocols. Equipment can be decontaminated with approved chemical or heat treatments before and/or after use (Wolfel et al., 2015). Military use of field laboratories also provides remote monitoring and on-site investigation of unusual disease outbreaks or terrorist attacks (Wolfel et al., 2015).

2.8.1 The Finnish Mobile Diagnostic CBRN Field Laboratory

The Finnish Defence Forces have developed and introduced Operational CBRN Defence Units for national defence. The Diagnostic Deployable CBRN Laboratory has been designed and established for international missions as well as domestic use (Kinnunen et al., 2012, Siekkinen et al., 2012), and the unit has been evaluated and approved by NATO.
The Diagnostic Deployable CBRN Laboratory can be used for all three major tasks of the Finnish Defense Forces: 1. national defense of Finnish territory and population; 2.

support the Finnish authorities to secure the society, and; 3. aid international crisis management operations. The field laboratory can be used in a variety of defense purposes, including natural outbreaks and deliberate release incidents. The laboratory can be transported by land, sea or air, and is fully operational within 72h without external support (Figure 4). The trailer of the laboratory has been designed for laboratory operation in all climate conditions with its own electricity, water and fuel supply. The field laboratory includes four separate modules that analyze chemical (C), biological (B), radiological/nuclear (RN), and field hygiene (FH) samples (Figure 5). The B laboratory is designed to identify pathogens in biological samples and satisfies BSL-3 requirements. Under normal circumstances, a negative air pressure (-30 Pa) is maintained in the B laboratory, but in the case of a CBRN-related situation or in the presence of smoke or dust, the interior can be pressurized to +50 Pa relative to the outside environment. The incoming air is CBRN-filtered, and an air conditioning system maintains air temperature at a desired level. Exhaust air is filtered through double high efficiency particulate air filters (Kinnunen et al., 2012, Siekkinen et al., 2012).

Figure 4. The Diagnostic Deployable CBRN Laboratory of the Finnish Defense Forces can be transported also by air (Deployable CBRN Field Laboratory transported by air, reprinted with the kind permission of the Finnish Defence Forces).

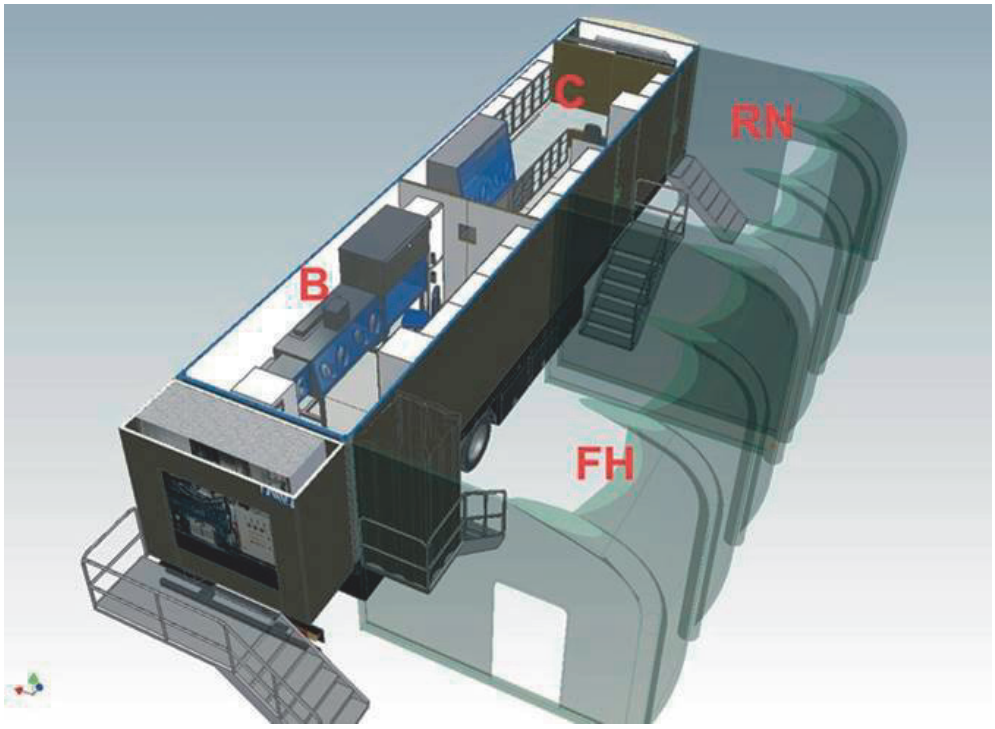


Figure 5. The Diagnostic Deployable CBRN Laboratory of the Finnish Defense Forces includes four separate laboratory modules, analyzing chemical (C), biological (B), radiological/nuclear (RN), and field hygiene (FH) samples (Deployable CBRN Field Laboratory, reprinted with the kind permission of the Finnish Defence Forces).

The B laboratory also includes a microbiological safety cabinet (MSC) line. The two MSCs (class III and II) are connected by a dunk tank located under the benchtops. The B laboratory includes all necessary analytical instruments including equipment for sample preparation, real-time PCR thermocyclers for sensitive nucleic acid detection, and immunoanalyzers for rapid antigen and antibody detection. Sample material is subjected to nucleic acid extraction which inactivates biological agents. The purified nucleic acid preparations are then transferred through the dunk tank to the class II MSC for setting up PCR testing and subsequent analysis, whereas used equipment and waste are sterilized using the pass-through autoclave. Surface decontaminants, hydrogen peroxide fumigation, and an autoclave are used for decontamination and waste disposal. In addition to rapid field detection, the field laboratory may be used for collection of forensic evidence related to possible cases of alleged use of biological agents (Kinnunen et al., 2012, Siekkinen et al., 2012).

2.8.2 The CBRN reconnaissance team and the forensic sampling team

The field laboratory capabilities are combined with the field experience and expertise of the CBRN reconnaissance, decontamination and sampling team and associated medical personnel. The field laboratory and its crew can collect operational and forensic samples and perform analyses and identification of chemical, biological and radiological agents. The laboratory personnel work closely with the team responsible for forensic sampling and identification of biological, chemical and radiological agents (SIBCRA team) (Figure 6). The team unit consists of a leader and specialists in C, B, or RN, depending on the mission. In forensic sampling, documentation and sample chain-of-custody play critical roles while evidence quality is determined by sample collection and processing and by their demonstrable link to the CBRN event (Kinnunen et al., 2012).

Figure 6. The CBRN-reconnaissance team investigating an unknown sample during a military exercise (The CBRN-reconnaissance team, reprinted with the kind permission of the Finnish Defence Forces).

2.9 Rapid point-of-care diagnostic tests

Rapid identification and accurate characterization of a pathogen play a key role in the initiation of appropriate control measures and medical treatment (Irenge and Gala, 2012, Ivnitski et al., 2003). Furthermore, Nouvellet et al. (2015) estimated that rapid diagnostic tests in combination with confirmatory testing could have reduced the scale of the 2013–2015 ebola epidemic in West Africa by over a third.

Several diagnostic tools are available for the detection and identification of biological agents, e.g., plate culture, molecular methods, immunofluorescent and gram staining, antigen detection with immunoassays, and serological techniques (Grunow et al., 2000). However, relatively fast diagnostic tests, such as those based on immunoassay chemistry, often have limited sensitivity (Irenge and Gala, 2012, Zasada et al., 2015) while others, such as conventional plate culture, require specialized microbiological laboratories and lengthy processing times to obtain conclusive results (Grunow et al., 2000, Hatchette et al., 2009).

Robust and reliable equipment for the detection and characterization of pathogens is often large, heavy and in other ways unsuitable for mobile diagnostics. Transporting samples to a fixed-location laboratory may take several hours or even days. A field-ready and reliable diagnostic response capability is urgently required for use at the point of first detection or centre of exposure in order to identify the causal agent, provide primary care, and monitor and control the spread (Inglis, 2013).

2.9.1 Antigen detection tests

Immunoanalytical methods are based on the specific affinity between microbial antigens and antibodies. These methods can be applied in the rapid detection of bacteria, viruses and toxins and have been developed primarily for point-of-care (POC) diagnostics and field testing. Some of the antigen detection tests require powered automatic instruments (Jokela et al., 2015, Gunell et al., 2016). These tests have been suggested to be more sensitive (Jokela et al., 2015) than commonly used lateral-flow tests (de la Tabla et al., 2010, Irenge and Gala, 2012, Zasada et al., 2015), and have been applied in healthcare centers (Gunell et al., 2016).

Lateral-flow tests have been developed primarily for rapid field diagnostics, but can also be useful for clinical laboratories (Ferris et al., 2009, Nouvellet et al., 2015). Lateral-flow tests are small strips and easily adaptable for field use and biothreat protocols. The advantage of the strip tests are their user-friendly format, short processing time and long-

term stability over a wide range of climate conditions and they are also relatively inexpensive to manufacture (Ferris et al., 2009, Liu et al., 2016, Nouvellet et al., 2015, Zasada et al., 2015). These single-use assays are based on immunochromatographic or immunoenzymatic techniques. Colored end-products confirm or reject the presence of the target. Antigen-detection assays are based on an antibody-coated membrane, and use capillary flow for the elution of labeled antibodies conjugated with target antigens in the liquid phase. The complex formed by the antigen and the labeled antibodies migrates along the test membrane and binds to the stationary antibody in the test membrane, forming a visible result line. The test strip also contains an integrated control system with a line to indicate a complete and successful assay (Figure 7) (Lee et al., 2013, Parolo et al., 2013).

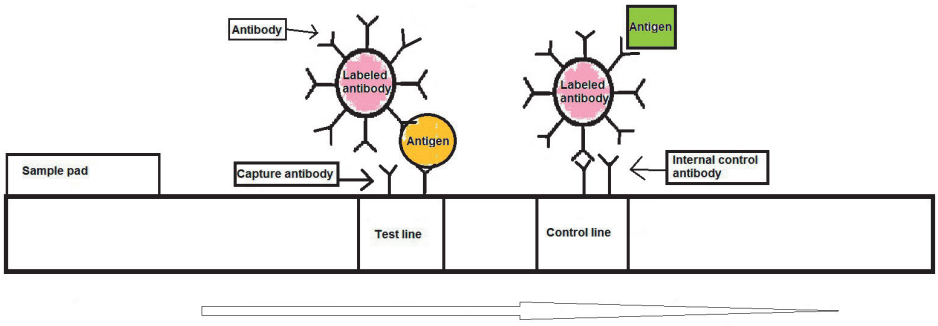


Figure 7. A complex formed by the sample antigen and labeled antibodies migrates through an antibody-coated membrane via capillary flow. The strip includes test and control lines, and results (both test and control) are read as visible lines.

Although these assays are easy and fast to perform, they sometimes lack sensitivity (Irenge and Gala, 2012, Zasada et al., 2015). Thus, infected individuals may be misdiagnosed and returned to the population without further treatment (Nouvellet et al., 2015). However, the rapid assays may be useful for initial screening for the presence of biological agents (Ferris et al., 2009). Positive results can be further confirmed to exclude false-positive results by more sensitive gold standard tests, such as PCR or other nucleic acid detection tests (Nouvellet et al., 2015). Lateral-flow tests have been developed by many companies for biological threat agents, respiratory viruses and several toxins (Kanwar et al., 2015, Mirski et al., 2014, Rodriguez et al., 2015, Sambursky et al., 2006).

Due to suboptimal sensitivity of some of the rapid immunoassay detection tests available, nucleic acid detection assays are becoming increasingly important for fast, sensitive and specific identification of pathogens (Grunow et al., 2000, Hatchette et al., 2009, Irenge and Gala, 2012). Therefore, rapid immunoassay detection tests confirmed with real-time PCR have been suggested to be an effective combination, and can be employed in the field to control an epidemic by quickly isolating and treating infected patients (Nouvellet et al., 2015).

2.9.2 Nucleic acid detection tests

Nucleic acid detection tests such as the PCR are gold standard methods for the detection of biological agents due to their high sensitivity and specificity (Grunow et al., 2000, Hatchette et al., 2009, Mackay, 2004, Pohanka and Skladal, 2009). The PCR technique faithfully copies, or amplifies, a nucleic acid segment many thousands or millions of times which can then be detected by different means (Almassian et al., 2013). PCR relies on a thermal cycle program in which the target region is repeatedly denatured and copied by a thermostable polymerase guided by region-specific oligonucleotide primers.

Real-time PCR is a new approach compared to standard PCR, where small aliquots of the amplified PCR product are transferred to agarose gels containing a fluorescent stain and allowed to migrate in an electric field (electrophoresis) prior to visualization with UV transillumination. In contrast, real-time PCR offers simultaneous amplification and quantification of a target nucleic acid region with a template-specific fluorescent probe added to the reaction buffer. Specific primers and a fluorescent probe reveal if the sample contains nucleic acid belonging to the target pathogen (Mackay, 2004).

Primers containing sequences complementary to the target region along with a polymerase enzyme are the key components, which enable selective and repeated amplification (Almassian et al., 2013). Primers are chemically synthesized oligonucleotides, with a length of about twenty nucleotides and are hybridized to target DNA, tens to hundreds of bases apart from each other.

A probe, used in real-time PCR, is a short DNA fragment used to detect the presence of a complementary target. The probe thereby hybridizes to single-stranded DNA which is a perfect match (Almassian et al., 2013). Minor groove binding (MGB) probes consist of a fluorophore ("reporter") covalently attached to the 5'-end and a "quencher" at the 3'-end. As long as the fluorophore and the quencher are in proximity, i.e., in the non-hybridized state, quenching inhibits any fluorescence (Figure 8). The benefit of MGB probes are their short length and specificity (Kutyavin et al., 2000).

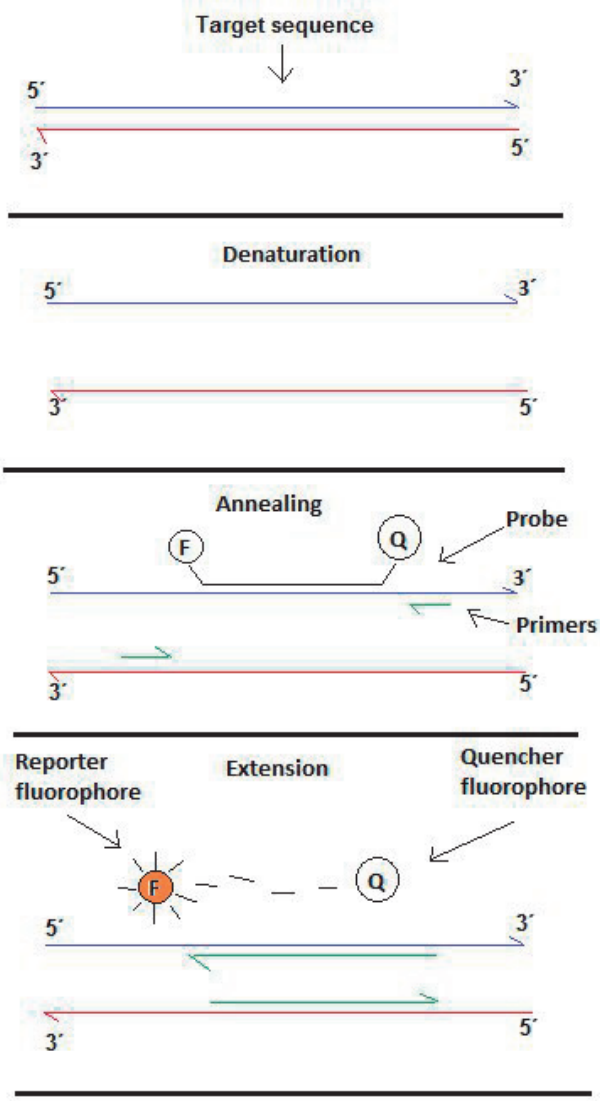


Figure 8. Principle of real-time PCR. Reporter fluorophore and a quencher are attached to a probe (i.e., complementary sequence) to the target pathogen DNA marker. When the target DNA is amplified, the reporter fluorophore is separated from the quencher and can be detected by its fluorescence.

A PCR mix consists of a polymerase, a buffer solution, specific primers and probe, deoxynucleotides, and a nucleic acid template from the sample under investigation. A RNA template has to be transcribed into complementary DNA (cDNA) with a reverse transcriptase prior to amplification (Almassian et al., 2013).

In addition to real-time PCR, other alternative nucleic acid detection tests have been developed. Isothermal amplification technology is also a sensitive and suitable method for portable gene amplification detection (Eboigbodin et al., 2016, Eboigbodin and Hoser, 2016). This novel technology performs amplification at a stable temperature as compared to conventional and real-time PCR. Thus, without thermal cycling, this technology needs less power, which represents a distinct advantage in the field (Almassian et al., 2013). Of gene-amplification methods used in the field, real-time PCR is the gold standard but isothermal amplification is a promising technology for the near future. Simple real-time interpretation of test results makes the technology a strong candidate for future point-of-care diagnostics, especially in situations where the user does not have special expertise. Commercially available nucleic acid detection tests usually include both pathogen-specific kits and the instrument (Eboigbodin et al., 2016). Some products also include an automated sample preparation method (Guenaoui et al., 2016, Hirvonen et al., 2015, Moussa et al., 2016, Zumla et al., 2014).

2.9.2.1 Sample preparation methods

Isolation of nucleic acid from biological samples is the first step prior to nucleic acid detection tests. Such methods perform optimally when purified DNA or RNA (i.e., free from potential amplification inhibitors) is used (Shaw et al., 2009). Clinical samples are inactivated and prepared for genetic amplification. Preparation includes nucleic acid extraction and purification from other material in the sample. During nucleic acid extraction and purification, any infectious pathogens are inactivated. Extracted and purified nucleic acids are then used as template in gene amplification protocols. Both automated extraction instruments and manual extraction kits are widely available and used routinely in industrial, medical and academic settings (Dauphin et al., 2011, Ip et al., 2015, Whitehouse and Hottel, 2007).

2.9.3 Genetic characterization of pathogens

Genetic characterization includes sequencing and bioinformatics and is an important part of detecting and identifying pathogens prior to mounting an appropriate response. Information concerning the genetic profile of a pathogen(s) can help to distinguish natural outbreaks from a deliberate release (Cheung and Kwan, 2012, Chin et al., 2011, Dembek et al., 2007, Koser et al., 2012). Genetic characterization helps trace the source of a pathogen and its transmission pathway by comparing the prevalent strain in the outbreak to those circulating in nearby populations. It also helps to investigate any ge-

netic manipulation, mutations, geographic patterns, and in relation to known laboratory strains or unexpected antibiotic resistance (Sjodin et al., 2013). With genetic characterization, it is possible to quickly identify genome differences between closely related isolates.

Commercial high-throughput techniques based on Sanger sequencing (i.e., the use of fluorescent di-deoxynucleotide terminators) have been available for decades, but they have their limitations in the form of slow processing speeds, high cost and high demand of operator skill (Mardis, 2008, Sanger et al., 1977, Torok and Peacock, 2012). Sanger sequencing is not easily adapted for processing large genomes or large numbers of samples, when at the same time recent developments of sequencing technologies have made it possible to rapidly sequence the entire genome of a pathogen (McGinn and Gut, 2013, Torok and Peacock, 2012).

Nowadays, next generation sequencing (NGS) technologies in concert with genome sequence libraries available in public databases are capable of providing whole-genome sequence data (Vernikos et al., 2015). Whole-genome sequencing (WGS) is an emerging technology with considerable promise for diagnostic and public health microbiology (Torok and Peacock, 2012). WGS technologies have become more reliable and are being applied in clinical microbiology laboratories to detect and characterize pathogens (Bertelli and Greub, 2013, Quinones-Mateu et al., 2014, Van den Hoecke et al., 2015). Some NGS platforms such as the Illumina MiSeq and Life Technologies Ion Torrent can provide WGS data within 2–3 days of receiving a sample (Van den Hoecke et al., 2015).

Due to the recent developments in sequencing technology, genetic characterization methods are accessible and affordable (Quick et al., 2014). Small size nanopore sequencing technology is a single-molecule detection system and operates by driving molecules electrophoretically in solution through a nano-scale pore (Branton et al., 2008). The technology has been used successfully to sequence the entire genome of some influenza A viruses (Wang et al., 2015) and has also been used in the field to sequence the ebola pathogen during a recent outbreak in West Africa (Gardy et al., 2015). This field-ready technology reduces the need to ship biological samples to off-site diagnostic laboratories and thereby minimize response time.

The challenge with whole-genome sequencing methods is the lack of high-quality reference databases containing pathogen genomes (Torok and Peacock, 2012). Furthermore, accessing and comparing an unknown sequence to an on-line database could prove challenging in a remote location with poor telecommunications coverage (Quick et al.,

2014). Also, accuracy of the portable nanopore sequencing technology has recently been reported to be between 65 to 85 % per base (Kilianski et al., 2015)

3. AIMS OF THE STUDY

The aims of this study were to develop and test the performance of portable real-time PCR systems in the detection of pathogenic biological agents in the field, to further complement the workflow by characterization of the pathogen, and to present the critical factors in a successful investigation of biological agents in biothreat preparedness.

The specific aims of this study were:

- To test and compare the performance of commercially-available rapid nucleic acid extraction methods to be used in the field with portable thermocyclers.

- To develop highly sensitive and rapid on-site methods for the detection of three high-risk biothreat bacterial pathogens (*Francisella tularensis*, *Bacillus anthracis* and *Yersinia pestis)* under field conditions.

- To develop a method that detects all subtypes of influenza A virus and a method for the specific detection of influenza A(H1N1)pdm09 virus with rapid identification technology in the field.

- To genetically characterize adenovirus serotypes circulating in conscripts of the Finnish Defence Forces in order to gain detailed genetic characteristics of the virus. Sequence data of a biothreat pathogen can reveal possible deliberate release.

4. MATERIALS AND METHODS

This section briefly describes the materials and methods used in this study. More detailed descriptions can be found in the original publications, which are referred to here by the Roman numerals I-IV.

4.1 Materials

4.1.1 Bacterial and tissue samples (I, II)

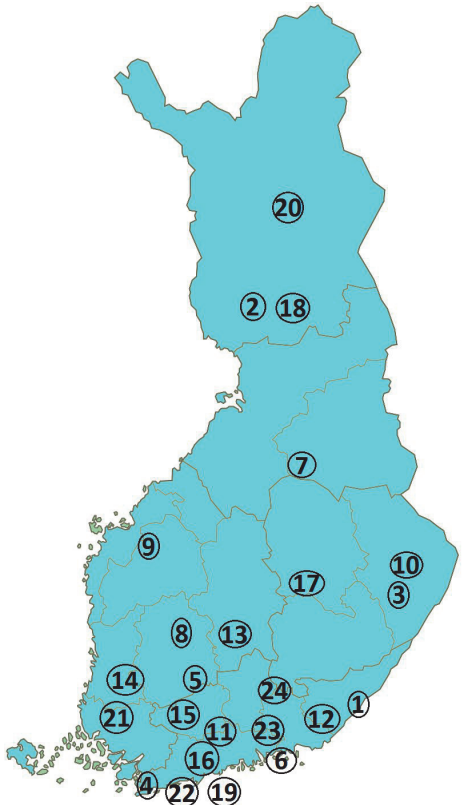
Bacterial control samples for specificity testing of real-time PCR assays were received from the bacterial collection of the Centre for Biological Threat Preparedness laboratories (I, II). Specificity testing was done with 24 bacterial strains (II) (Table 6). In addition, 1035 vole liver tissue samples were received from the Finnish Forest Research Institute (nowadays the Natural Resources Institute of Finland) collected at the Suonenjoki Research Station of the Finnish Forest Research Institute and at the Kilpisjärvi Biological Station of the University of Helsinki (II).

Table 6. Specificity of the bacterial assays was tested with DNA of 24 bacterial strains.

Bacterial strains used for real-time PCR specificity testing
Agrobacterium tumefaciens C58C1/RP4
B. anthracis ATCC 4229 (pXO1-/pXO2+)
B. anthracis Sterne 7702 (pXO1+/pXO2-)
B. cereus ELMI 21
B. licheniformis ELMI 325
B. mycoides ELMI 44
B. thuringiensis ELMI 123
B. thuringiensis subsp. *kurstaki-aizaway*
Brucella melitensis 72
Enterobacter cloacae tks461
Escheria coli C600/pYET6
F. tularensis LVS (ATCC 29684)
Moraxella catarrhalis 035E
Staphylococcus aureus ATCC 25923
Y. bercovieri 3016/84
Y. enterocolitica 1309/80
Y. intermedia 9/85
Y. kristensenii 119/84
Y. mollaretii 92/84
Y. pestis EV76-c
Y. pestis KIM D1
Y. pseudotuberculosis H305-36/89
Y. pseudotuberculosis No.90
Y. ruckeri RS41

4.1.2 Viral samples (III, IV)

Nasopharyngeal aspirates or combined flocked nasal and throat swab samples were collected by medical personnel from military conscripts with acute respiratory infections. These specimens were intended for sentinel surveillance of respiratory infections conducted by the Institute of National Health and Welfare (THL) from 24 military garrisons and border guard detachment units (III, IV) (Figure 9).



1. *Bgd of South East Finland
2. *Bgd of Lapland
3. *Bgd of North Karelia
4. Dragsvik
5. Halli
6. Hamina
7. Kajaani
8. Keuruu
9. Kauhava
10. Kontiolahti
11. Lahti
12. Lappeenranta
13. Luonetjärvi
14. Niinisalo
15. Parola
16. Riihimäki
17. Rissala
18. Rovaniemi
19. Santahamina
20. Sodankylä
21. Säkylä
22. Upinniemi
23. Utti
24. Vekaranjärvi

Figure 9. Respiratory samples were collected from army recruits or staff of 24 military garrisons or Finnish boarder guard departments (*).

Nucleic acids isolated from influenza A viruses, adenoviruses and from other common respiratory viruses (i.e., influenza B, influenza C, parainfluenza virus types 1, 2, and 3, human respiratory syncytial virus, human bocavirus and human metapneumovirus) from the virus collection of the THL virus laboratories were used in sensitivity and specificity testing of the real-time PCR methods (III). The specificity of the assays was tested with RNA from 18 influenza A viruses, as well as with nine other common respiratory viruses (III) (Table 7). In addition, RNA from 125 influenza A samples (III) and DNA from 837 adenovirus samples (IV) were analyzed.

Table 7. Specificity of the influenza A assays was tested with nucleic acid of 27 viral strains.

Virus strains used for real-time PCR specificity testing
Influenza A, A/Panama/2007/99 (H3N2)
Influenza A, A/Fin/81/08 (H3N2)
Influenza A, A/Fin/149/08 (H3N2)
Influenza A, A/Fin/209/08 (H1N1)
Influenza A, A/Fin/213/08 (H1N1)
Influenza A, A/Mallard/Neth/12/00 (H7N3)
Influenza A, A/HK/1073/99 (H9N2)
Influenza A H5N1 Clade 1
Influenza A H5N1 Clade 2.1
Influenza A H5N1 Clade 2.2
Influenza A H5N1 Clade 2.3.2
Influenza A H5N1 Clade 2.3.4
Influenza A, A/Fin/544/09 (H1N1)pdm09
Influenza A, A/Fin/571/09 (H1N1)pdm09
Influenza A, A/Fin/577/09 (H1N1)pdm09
Influenza A, A/Fin/579/09 (H1N1)pdm09
Influenza A, A/Fin/582/09 (H1N1)pdm09
Influenza A, A/Fin/554/09 (H1N1)pdm09
Influenza B
Influenza C, C/Ann Arbor/1/50
Parainfluenza virus 1
Parainfluenza virus 2
Parainfluenza virus 3
Respiratory syncytial virus
Bocavirus
Human metapneumovirus
Adenovirus (type 7)

4.2 Methods

4.2.1 Sample preparation methods (I, II, III)

Sample preparation methods (e.g., nucleic acid extraction) to be used in field for bacterial and viral samples were tested and compared on the basis of their performance and features of the extraction kits (I–III).

Commercial DNA extraction kits were compared and also evaluated for their ability to inactivate *Bacillus thuringiensis* spores and to extract spore DNA from spiked powder samples (icing sugar and potato flour) (I). The kits employed different techniques, including heat treatment, spin column procedures, bead beating, and magnetic beads (Table 8).

Table 8. Commercially-available DNA extraction kits evaluated in this study.

DNA extraction kit				
	QIAamp DNA Mini Kit	**RTP Pathogen Kit**	**ZR Fungal / Bacterial DNA MiniPrep**	**genesig Easy DNA/RNA Extraction kit**
Method	Spin column	Spin column	Spin column	Magnetic beads
Equipment	Heat blocks	Heat block, thermomixer	-	Magnetic rack
Lysis method	Heat treatment	Heat treatment	Lysis solution and bead beating	Lysis buffer

The performance of the kits was evaluated based on the processing time, DNA recovery and purity, the need for additional laboratory equipment (i.e., heat block, vortex disruptor), the elimination of potential PCR inhibitors, and the sensitivity of the subsequent real-time PCR analysis (I). Also centrifugal filter units (Merck Millipore Ultrafree-MC Centrifugal Filter Devices) were tested for the additional removal of spores from DNA samples. Extracted DNA was analyzed with a real-time PCR assay optimized for *B. thuringiensis* (I).

To demonstrate the field capability and point-of-care suitability, nucleic acid extractions were also performed in field conditions (II, III). DNA from 1035 rodent samples was extracted and analyzed at the biological research stations in Kilpisjärvi and Suonenjoki in conditions outside a sophisticated laboratory (II) and RNA from 21 clinical samples

was extracted at a healthcare center of a military garrison (III). All nucleic acid extractions were performed according to the manufacturer's instructions (I–III).

4.2.2 Biosafety in sample preparation (I, II, III)

All pathogenic biological samples must be inactivated by chemical or physical means prior to analysis in order to protect laboratory personnel from infection (Blow et al., 2004), (Dauphin et al., 2009). For example, bacterial spores are highly resilient and may remain viable following standard inactivation protocols (I). Nucleic acid extracts should be non-infectious but given their pathogenic origin, appropriate personal protective equipment (PPE) was used throughout the study (Daugherty et al., 2010). In addition to carefully selected nucleic acid extraction methods (I–III), PPE was selected according to the pathogenic risk assessment protocol (i.e., coverall suit, goggles and FFP-3 facemask, gloves and boots) (Grolla et al., 2011, Daugherty et al., 2010). The use of PPE is emphasized according to the risk group of the pathogen.

4.2.3 Real-time PCR assay design and optimization (I, II, III, IV)

Nucleic acid samples of the pathogens were amplified and identified with specific primers and probes using portable real-time thermocyclers (II, III). In addition to Taqman probe-based chemistry, SYBR-green chemistry was used for the identification of adenovirus serotypes (IV).

A novel real-time PCR assay for the detection of *F. tularensis* was developed (II). The assay target was the insertion sequence element ISFtu2, which is well represented in the *F. tularensis* genome. Other oligonucleotides used in this study have been reported previously (I–IV), and combinations were based on sequences available in the NCBI database. The ISFtu2 assay was developed using the Primer Express software, version 2.0 (Life Technologies Ltd, Carlsbad, CA, USA) (II) and the oligonucleotide concentrations for real-time PCR were optimized (II). Furthermore, commercial real-time PCR kits were evaluated for field use. The final kit selection was based on performance and features, e.g., highest sensitivity based on threshold cycle (Ct) values and fluorescence levels (II, III).

4.2.4 Sensitivity and specificity testing of the real-time PCR assays (II, III)

Sensitivity and specificity of the real-time PCR assays were tested in a reference laboratory (THL) both with portable thermocyclers and with gold standard real-time PCR equipment Applied Biosystems (ABI) 7300, and results were compared (II, III). Sensi-

tivity of the real-time PCR assays was tested using serial ten-fold dilutions of nucleic acid extracted from bacterial (II) and viral (III) strains. The specificity of the bacterial assays was evaluated with DNA extracted from clinical and environmental bacterial samples (II), and the specificity of the viral assays was tested with RNA extracted from influenza A viruses and nine other common respiratory viruses (III). Real-time PCR assays were performed in duplicate as part of a dilution series and each reaction was carried out with optimized oligonucleotide concentrations (II, III).

4.2.5 Controls in DNA amplification (I, II, III, IV)

Each PCR assay included a negative (i.e., no template) control (NTC) and a positive nucleic acid control (I, II, III, IV). An internal positive control (IPC) was used in the confirmation assay performed on the ABI 7300 (III). IPC in DNA amplification is used to monitor the performance of real-time PCR. The IPC ensures that a failed real-time PCR is not mistaken for a negative test result. The IPC distinguishes between two types of negative reactions; samples identified as negative because (1) they lack the target sequence or (2) due to the presence of PCR inhibitors. PCR inhibitors can originate from the sample itself or may be introduced during sample processing or nucleic acid extraction, usually causing decreased sensitivity or false negatives. PCR inhibitors can be present in different types of matrices, such as clinical, food or environmental specimens (Schrader et al., 2012). An IPC mixture consists of a specific IPC template as well as specific primers and a probe, which is labeled with a different fluorophore (e.g., VIC) than the probe specific to the sample DNA (e.g., FAM). Different fluorophores enable simultaneous detection of both target DNA and IPC DNA. Failed IPC amplification usually reflects the presence of PCR inhibitors.

4.2.6 Carrying out a real-time PCR analysis in field (II, III)

Portable real-time PCR thermocyclers and pathogen-specific assays were used to determine if pathogenic agents could be rapidly and reliably diagnosed in the field (II, III). Size, weight and robustness of thermal cyclers are important technical issues for the development of real-time PCR methods for use outside of the laboratory (Ivnitski et al., 2003). The PikoReal Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA) weighs 10 kg (II) and RAZOR EX Instrument (BioFire Defense, Salt Lake City, UT) 4.9 kg (III), and both instruments are comprised of robust technology suitable for use in the field. With both real-time PCR instruments, nucleic acid amplification occurs in a closed reaction plate (II) or a pouch (III) and this separates the amplified product from the hardware and the environment, thus limiting the risk of contamination of amplified

DNA fragments which may lead to false positives. The PikoReal cycler was packed into a compact shock- and water-resistant Pelican case (Pelican Products Inc., Torrance, CA) together with a laptop computer with PikoReal software and conductor rails for a power supply (Figure 10). The DNA extraction equipment was transported in a separate case (Figure 11).

Figure 10. The PikoReal technology equipment was packed into a compact Pelican box with all necessary hardware.

Figure 11. The DNA extraction equipment was packed in a separate case.

To prevent contamination, different solutions (i.e., mechanical and chemical barriers) can be used (Aslanzadeh, 2004). In this study, all field diagnostic stages (Table 9) were done in separate spaces and a set of positive, non-template and extraction controls was used with each assay (II, III). This protocol was implemented both in biological research stations (II) and healthcare centers of military garrisons (III).

Table 9. To avoid contamination, all field test processes should be done in separate spaces.

Field diagnostics stages
1. Sampling
2. Nucleic acid extraction
3. Preparation of qPCR master mix
4. Adding of template
5. Amplification and detection of nucleic acid and interpretation of results

Real-time PCR contamination is the accidental introduction of "foreign" material, e.g., amplified DNA or laboratory personnel DNA, and can significantly distort results. The risk of contamination is high under field conditions due to nature of working spaces and ability to maintain an appropriate level of hygiene for each of the different stages (Hedman et al., 2013, Aslanzadeh, 2004). Contamination can occur at each stage of a

real-time PCR analysis, even in sample collection. Positive control material and real-time PCR products already present in the laboratory are the most common sources of contamination. The quantity of amplified nucleic acid after real-time PCR is enormous compared to the starting material. Therefore, tubes or plates containing real-time PCR products were never opened following amplification. The advantage of real-time PCR, compared to conventional PCR, is that amplification and quantification occur simultaneously, removing the need to expose PCR product to the workspace.

4.2.7 Genetic characterization of adenoviruses by sequence analysis (IV)

During five years from 2008 to 2012, 3577 respiratory specimens were collected from Finnish military conscripts presenting symptoms compatible with acute respiratory tract infection. A total of 794 of these specimens were identified as adenovirus-positive and were available for genotype identification (IV). For 672 of these specimens, the serotypes were successfully determined by DNA sequencing. Twelve samples which gave inconclusive results from direct sequencing of the amplicon were further analyzed by cloning. PCR amplicons were cloned using the TOPO TA cloning kit according to the manufacturer's instructions and subsequently amplified in *Escherichia coli* (Invitrogen Corporation, Carlsbad, CA, USA). Amplicons and cloning products were sequenced by the Finnish Institute for Molecular Medicine (Helsinki, Finland) and nucleotide sequences were further analyzed with the Sequencer 5.1 program (Gene Codes Corporation, Ann Arbor, MI USA). The BLAST search tool and ClustalW were used to compare amplicon sequences to reference sequences published in GenBank (IV).

5. RESULTS AND DISCUSSION

5.1 Evaluation of nucleic acid extraction methods in the field (I, II, III)

The selected extraction kits relied on different protocols and techniques, including heat treatment, spin column filtration, bead beating, and magnetic beads. These translated to differences in processing time and performance (I–III), i.e., sensitivity differences of the real-time PCR (I). The C_t values varied from 18.4 to 19.4. The limit of detection of the extracted *Bacillus thuringiensis* DNA varied from 3 x 10^1 to 3 x 10^3 colony-forming units (CFU) and reflected DNA yields (Table 10) (I). The extraction time used for nine samples varied from 65 to 155 min (Table 10) (I). Overall, the manual extraction kits gave consistent results in real-time PCR. The differences in cost per sample between the kits tested did not correlate with the overall performance (I). Also, centrifugal filter units removed spores from DNA extracts (bacterial spore positive) and from control samples known to contain bacterial spores. Thus, this additional step is worthwhile in field situations where it is of utmost importance that samples are spore-free (Table 10) (I).

Table 10. Performance indicators were established with commercial extraction kits.

	DNA extraction kit				
Extraction kits		**QIAamp DNA Mini Kit**	**RTP Pathogen Kit**	**ZR Fungal / Bacterial DNA Mini-Prep**	**genesig Easy DNA/RNA Extraction kit**
Performance indicators	**Spore inactivation**	Yes	Yes	Yes	No*
	Processing time	155 min	100 min	65 min	105 min
	DNA yield	34 ng/µl	78 ng/µl[6]	17 ng/µl	41 ng/µl
	DNA purity (A260/A280)	1.8	2.5	1.5	1.4
	Ct value	18.4	18.7	19.0	19.4
	Limit of detection	3 x 10^1 CFU	3 x 10^1 CFU	3 x 10^3 CFU	3 x 10^1 CFU

*Centrifugal filter units removed the spores both from extracted DNA samples and from bacterial spore positive control samples.

The extraction methods used in the field were rapid and results obtained were reliable and comparable to those achieved in the reference laboratory (II, III). Some differences in DNA purity and yield were seen but the performance level of all extraction kits tested was considered suitable for field use (I–III). Furthermore, no contaminations were experienced in the field, even though conditions were suboptimal, i.e., working conditions were not validated compared to those in the reference laboratory (II, III). Extraction protocols were not performed in a portable glove box, which would represent an additional level of biosafety precaution. Commercially-available glove boxes designed for field-use are readily available (Grolla et al., 2012, Panning et al., 2007, Wolfel et al., 2015), and this or a small plastic tent could be used with positive controls to minimize the risk of cross contamination.

Sampling and pre-test sample handling are laborious and time-consuming steps in pathogen identification. Easy-to-use nucleic acid extraction methods are needed for point-of-care diagnosis and for inactivation of pathogenic agents in biothreat preparedness and response protocols. For this study, sample extraction steps were optimized according to preliminary testing and prior experience of their use e.g., storage capabilities, extraction time and usability of the kits in the field (II, III). The reagents of the nucleic acid extraction kits did not require a freezer or cold storage (cold chain), and it is therefore possible to store and use the kit at room temperature and in the field (I–III). Reagent stability and optimized protocols enable fast isolation of nucleic acids in the field (I–III).

In addition to manual kits intended for a small number of samples, automated extraction systems are also available for the rapid processing of large number of samples (Dauphin et al., 2011). However, limitations of the field dictate that a portable, low-volume and versatile method is preferable (I) and, compared to manual kits, the equipment necessary for automated extraction is more expensive to purchase, maintain and operate, requires a continuous and reliable power source, and contamination issues are more difficult to resolve. Thus, small systems that can be powered from portable power-supplies have clear advantages in field situations.

5.2 Analysis of the real-time PCR and genetic characterization results (I, II, III, IV)

5.2.1 Analysis of the real-time PCR results

The performance of newly-developed portable real-time PCR thermocyclers (PikoReal and RAZOR EX) was compared to a standard real-time PCR thermocycler, (ABI 7300). The PikoReal was tested for the detection of three high-risk biothreat bacterial pathogens (*Francisella tularensis*, *Bacillus anthracis* and *Yersinia pestis*) and the RAZOR EX for rapid on-site diagnostics and for reliable identification of influenza A virus. Performance of the assay was the same with all instruments, suggesting a robust technology that can be successfully applied in the field (II, III).

Sensitivity of the biothreat bacterial assays (*F. tularensis*, *B.anthracis* and *Y.pestis*) ranged from 1 to 100 fg of DNA, and no cross-reactivity was seen between species-specific assays nor with DNA extracted from 24 other bacterial species used as templates (II).

When analyzing 1035 rodent samples at the research stations in Suonenjoki and Kilpisjärvi, real-time PCR results using the PikoReal instrument were achieved in approximately 1.5 hours. Pre-PCR preparations including DNA extraction required approximately 45 min. Results from the field (i.e., screening for *F. tularensis* at the research stations with the PikoReal) agreed with the results obtained using both ABI 7300 and PikoReal real-time PCR instruments in the reference laboratory at THL (II).

Detection limits of the influenza A(H1N1)pdm09 assay and the influenza A type-specific assay were 8.8 PFU (plaque forming units) of cultured A/Finland/554/09 virus and 0.15 PFU of cultured A/Panama/2008/99 (H3N2) virus, respectively. The specificity of the assays was tested with RNA from 18 influenza A viral strains, as well as with nine other common respiratory viruses (III). With respect to the broad-reacting influenza A assay, all subtypes tested gave a positive signal while none of the other respiratory viruses in the control panel were amplified. With respect to the influenza A(H1N1)pdm09-specific assay, all pandemic influenza strains were positive and no cross-reactivity was seen with other influenza A or other respiratory viral strains.

Furthermore, 104 combined nasal and throat samples collected from military recruits presenting with symptoms of febrile upper respiratory tract infection during the pandemic wave in the autumn of 2009 were analyzed. Of these, 65 were influenza A(H1N1)pdm09 positive and 39 negative using both influenza A assays. No difference

in sensitivity or specificity between the ABI 7300 and the RAZOR EX technologies was observed.

To demonstrate point-of-care suitability of the RAZOR EX technology, 21 clinical samples were tested under field conditions at a healthcare center in a military garrison. Reliable results were obtained in 1.5 hours. Real-time PCR results with the RAZOR EX were obtained within 45 min and an additional 45 min were required for the RNA extraction.

Sensitivity and specificity of the assays, as well as size and weight of the equipment are important factors to be taken into account when developing real-time detection solutions for field use. The real-time PCR performance achieved in the field did not differ from that in the diagnostic laboratory (II, III). PCR can be sensitive to inhibitors and contaminations. To avoid contaminations, all field diagnostic stages were performed in separate spaces. To monitor possible contaminations, a set of positive, non-template and extraction controls were included in each assay (II, III). Furthermore, with the confirmatory instrument platform (ABI 7300), an internal positive control (IPC) was used (III). No contamination or PCR inhibition was observed during the study.

Technical limitations often restrict the use of sensitive and reliable diagnostics outside of sophisticated laboratories. Standard real-time PCR instruments, such as ABI 7300 are often large and heavy (29 kg) and include technology such as a tungsten-halogen lamp which is unsuitable for field use (III). Portable thermocyclers used in this study, the PikoReal and the RAZOR EX were light and small, (10 kg and 4.9 kg, respectively) and employed field-suitable and robust technology.

5.2.2 Evaluation of the portable real-time PCR technology in field use

Rapid and accurate detection and identification of infectious agents is essential in the response to disease outbreaks and biothreats. Diagnostic analyses with portable real-time PCR systems proved to be simple and rapid, as reliable results were achieved in field trials in approximately 90–120 minutes from the beginning of sample preparation to the completion of the real-time PCR (II, III). These results demonstrate that these instruments combined with pathogen-specific assays perform as well in field conditions as they do in sophisticated laboratories. The technology used in this study provides high sensitivity combined with speed and can be performed near the patient (III) and in the field (II). Sample preparation is a time-consuming step of pathogen identification (I, II, III) but with field equipment the time from sampling to obtaining a result is minimized because the molecular diagnostic protocols can be completed on-site.

The portable methodologies tested are reliable, robust, and suitable for use in pathogen surveillance. Portable real-time PCR instruments have been described earlier (Arif et al., 2013, Koskela et al., 2009, Matero et al., 2011, Paixão et al., 2008, Pierce et al., 2010), but compared to these techniques the latest commercially-available instruments provide technical advances by facilitating multiplexing, and modules for absolute and relative quantification as well as melting curve analysis. These advances enhance the capabilities of the equipment for research purposes (III).

The portable nucleic acid amplification technology evaluated in this study presents a strong candidate for portable and rapid diagnostics, such as identification of respiratory disease-causing agents in healthcare centers, in military garrisons or of biothreat agents in crisis management operations. If the diagnostic technology is in routine use on a regular basis or during epidemics in military garrisons, the technology and protocols will be effectively used in crisis situations.

The technology could be developed further by reducing power demand to the point that it could be met with a high-capacity battery and without the need for a separate laptop computer. This would reduce the size and weight of the equipment and form the basis of a backpack laboratory. Also, easy-to-use features like simplified interpretation of the results and wireless data transfer would streamline information sharing with decision makers. Lyophilized or otherwise stabilized real-time PCR reagents and assay kits stored at room temperature would enhance field utility of the system.
A prototype product of lyophilized reagents which includes a lyophilized master mix, an internal positive control, influenza A -specific oligonucleotides (Molsa et al., 2012), and an external positive influenza A-specific control was developed and tested in the course of this study. The performance of liquid and lyophilized reagents was evaluated in relation to assay sensitivity with the PikoReal and the ABI 7300 real-time PCR instruments. The PikoReal platform exhibited similar assay sensitivity as the ABI 7300. In addition, our results showed that the sensitivity of the assays was similar both with liquid and lyophilized reagents when using the PikoReal system. Furthermore, shelf life of lyophilized reagents was tested at RT and -20° C (Figure 12).

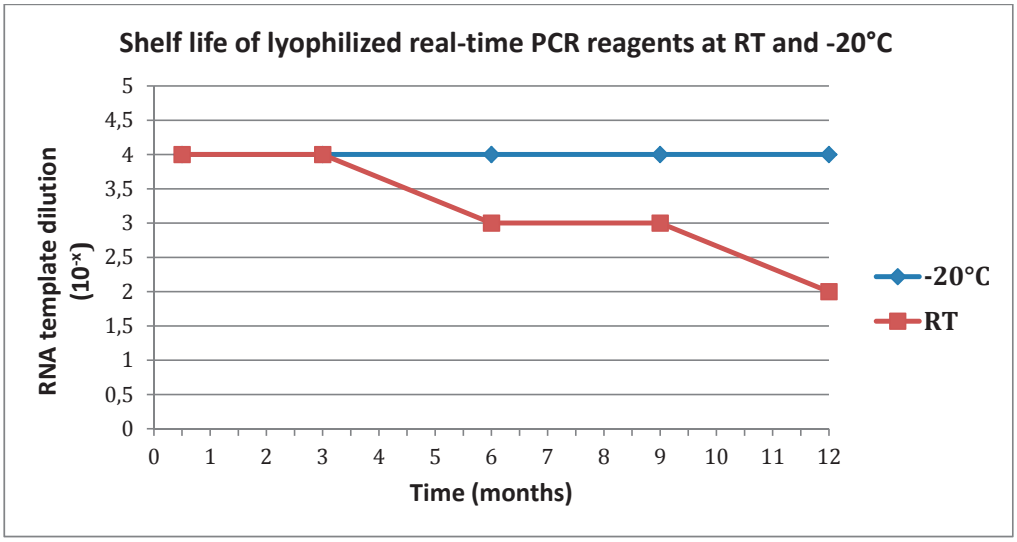


Figure 12. Lyophilized real-time PCR reagents stable at room temperature (RT) enhance the field utility of this system. Sensitivity of the lyophilized reagents, stored at RT or -20° C, were tested with real-time PCR during a period of twelve months. Lyophilized reagents were stable for three months in RT.

These results demonstrate the potential feasibility of a portable thermocycler and lyophilized reagents intended for rapid near-patient diagnosis of influenza A virus (M Mölsä et al. unpublished results). Assay development for other pathogens with lyophilized oligonucleotides as well as with lyophilized external and internal controls is in progress.

In addition to field diagnostics in this study (II, III), the portable diagnostic tool has been applied to be used in a crisis management operation of the Finnish Defence Forces (Figure 13). It has also been used under field conditions in Tanzania in the *Strengthening Health and Biosecurity in Tanzania by the Biodetection Capacity Building* project as a part of Finland´s contribution to the Global Health Security Agenda (Figure 14). These studies and the development of the diagnostic tool directly support Finland's commitment to the Biological Weapons Convention. Field diagnostic studies in Tanzania have shown that, in addition to limited and compromised workspace, high temperatures, humid conditions and the risk of contamination, as well as fluctuation in the electricity supply represent the main challenges to overcome in field conditions. This could be solved by using an uninterrupted power supply or a portable generator to drive those diagnostic instruments that are not battery powered (Inglis, 2013).

Figure 13. The portable thermocyclers were packed in a waterproof box with all necessary equipment to be used in an international crisis management operation.

Figure 14. The portable thermocyclers were set up for field use related to the project *Strengthening Health and Biosecurity in Tanzania by Biodetection Capacity Building.*

The possibility to develop and use in-house and other previously optimized assays in the field is considered an advantage. There are some commercial automated biodetection systems available, but these often work as closed systems, as they usually operate with commercial assay kits, and thus using optimized in-house assays is seldom possible. Also, access to the raw data, such as amplification curves and Ct values is often limited. This may, especially in cases of weak positive results, complicate decision-making and lengthen the response time unnecessarily. However, standardization and automation are important features which can enhance detection capabilities and reduce costs. They may also reduce detection error rates and improve the quality and reliability of diagnostic results. It is unlikely that automated systems of the near future will replace the need for confirmatory analyses provided by reference laboratories. Reference laboratories and in-field diagnostic capabilities complement each other and should not be considered separate entities.

The field-ready protocol presented here can be used to detect deliberately-released biological agents and is now applied in the mobile CBRN laboratory of the Finnish Defence Forces. Mobile field laboratories can help optimize field investigation work by applying a sampling strategy and specialized personnel to locations identified by the CBRN reconnaissance team. Diagnostic work can also be carried out in the field by CBRN reconnaissance or SIBCRA teams. Mobile laboratories can screen samples in the field with portable real-time PCR technology to reduce the number of samples to be sent to the reference laboratories for detailed analysis. Mobile laboratories are useful assets when collecting samples from or near the investigation site. The utility of mobile laboratories is recognized globally and was demonstrated by the World Health Organization's response to the latest Ebola outbreaks by teams of the ´European Mobile Lab´ in West Africa (Wolfel et al., 2015).

5.2.3 Genetic characterization of adenoviruses among Finnish Military Conscripts

One of the key challenges in investigating alleged use of biological weapons is the ability to differentiate intentional from naturally-occurring biological events, of which the former is identified for example by geographical occurrence of the pathogen or the presence of an atypical or genetically-engineered strain (Sjodin et al., 2013). Thus, there is a need for a scientific assessment of whether an outbreak is natural or deliberately caused by a hostile party (Sjodin et al., 2013). Molecular characterization of a pathogen is an important part of a strong response to biological threats as the origin(s) of the outbreak can be identified and an appropriate course of action followed. Genetic sequencing has been a reliable and robust method for over three decades (McGinn and

Gut, 2013) and amplicon sequencing has been presented as a viable approach for pathogen detection and characterization (Zumla et al., 2014). Nucleic acid sequencing has commonly been used for the identification of isolates and for their classification into genotypes that can be associated with host species or geographical regions (Gardy et al., 2015).

Respiratory infections, including adenovirus-associated acute respiratory illnesses are common among military recruits (Gray et al., 2000, Sanchez et al., 2001, Top, 1975). In this study, 672 specimens collected over a five-year period from 2008 to 2012 and identified as adenovirus-positive by real-time PCR were sero-typed by DNA sequencing to circulating in Finnish military garrisons (IV). Results suggested that major outbreaks during 2008–2012 were caused by adenovirus type 3 and type 4 (Figure 15), with one of the types dominating during each outbreak (Figure 16) (IV).

The serotype of 122 samples of the collected 794 adenovirus PCR-positive samples could not be determined with sequencing, possibly due to very low amount of viral DNA in the clinical sample. Most of the undetermined samples (27.8 %) and (21.1 %) were collected during 2011 and 2012, respectively. During these years samples were found to be PCR positive on initial testing with a probe-based real-time PCR, which yielded lower Ct-values than the SYBR Green assay, i.e., reflecting higher assay sensitivity. In this study, a SYBR Green-based PCR assay was used, as a probe assay may interfere with the sequencing of the PCR product.

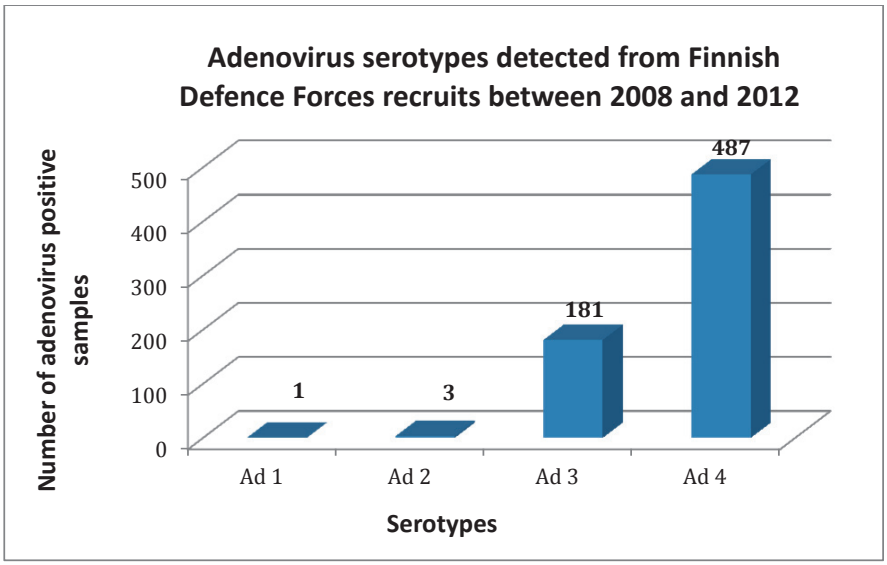


Figure 15. Adenovirus serotypes (Ad) 3 and 4 clearly dominated in adenovirus outbreaks in Finnish Military Garrisons during 2008–2012.

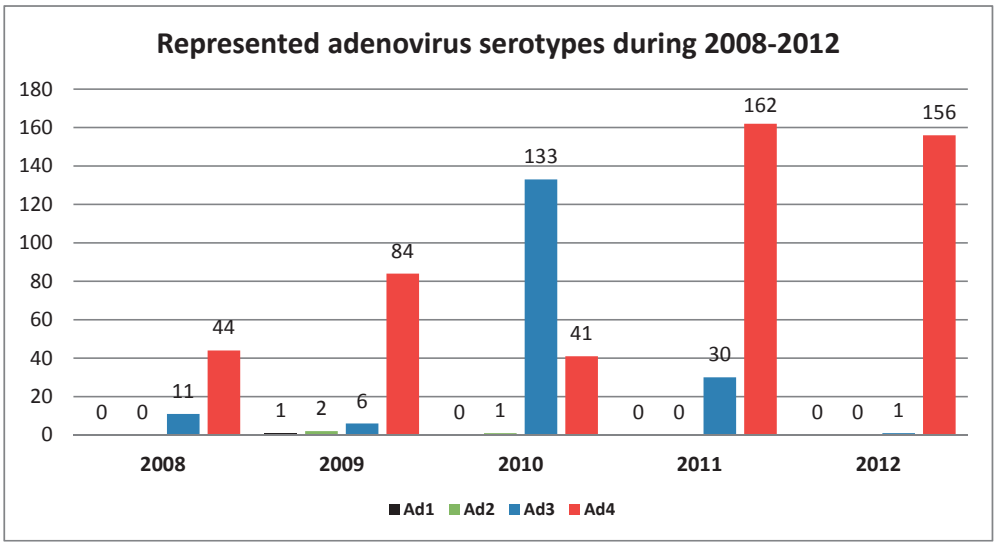


Figure 16. Either adenovirus serotypes (Ad) 3 or 4 were dominant in each outbreak during 2008–2012.

Both of the dominant adenovirus types (3 and 4) have caused large outbreaks and have been responsible for acute respiratory disease among military conscripts elsewhere (Kolavic-Gray et al., 2002, Kunz and Ottolini, 2010, McNeill et al., 2000, Top, 1975). A live oral vaccine against adenovirus types 4 and 7 was in use in the 1970s in US armed forces and was shown to reduce febrile respiratory illnesses in vaccinated recruits (Russell et al., 2006). After vaccine production was discontinued in 1996, a resurgence of adenovirus-induced respiratory illnesses among military recruits was noted (Kolavic-Gray et al., 2002, Russell et al., 2006). A new oral vaccine against adenovirus types 4 and 7 was approved in 2011 and the adenovirus vaccination program for military recruits resumed (Hoke and Snyder, 2013, Potter et al., 2012, Radin et al., 2014). Reintroduction of the adenovirus vaccination program in 2011 has resulted in a dramatic decline in infection and detection rates among recruits (Hoke et al., 2012, Radin et al., 2014). However, Gray et al. (2000) and Russel et al. (2006) suggested that during the years when infections by adenovirus types 4 and 7 were controlled by vaccination (1971–1996), adenovirus type 21 became the dominant strain causing infections. The information presented in this study may serve to help decide whether or not such a vaccine is likely to reduce adenovirus morbidity in the Finnish Armed Forces. It would, however, require continued monitoring to determine the extent to which other serotypes would replace adenovirus 4 should a conscript vaccination program be initiated.

5.2.3.1 Potential applications of portable genetic characterization methods

Identification and characterization of pathogens in clinical, veterinary, or environmental samples can help to clarify the origin and transmission pathway of a pathogen and provide information for dispersal modeling and epidemiology. Genetic characterization makes it possible to detect cases of genetic manipulation, mutation, geographic patterns, antibiotic resistance, unexpected infection source or virulence as well as match it to known laboratory strains that may have been adopted by weapons programs (Sjodin et al., 2013). The rapid availability of outbreak data also helps to direct epidemiological investigations (Dembek et al., 2007, Quick et al., 2014) and the development of rapid genome sequencing technology will soon provide this information in powerful detail (Quick et al., 2014). Novel nanopore technology shows potential for development of a miniature sequencer (Madoui et al., 2015, Quick et al., 2014), although the error rate must be improved before this technology can be considered reliable (Kilianski et al., 2015). Novel sequencing technology can provide new opportunities in infectious disease diagnostics, such as rapid sequencing in response to the early phase of a viral epidemic or the determination of viral genotypes during an unexpected outbreak in a remote location (Wang et al., 2015). Field-ready sequencing technology could be used as part of a real-time response to infectious diseases outbreaks, as the necessary equipment is highly portable and the protocols are simple, rapid and robust (Kilianski et al., 2015). Furthermore, real-time data of an outbreak could reveal key indicators of an emerging epidemic (Gardy et al., 2015, Quick et al., 2014).

Accuracy of genotyping improves as more sequencing data are made available and a consensus sequence is formed. Furthermore, data can be shared through on-line "cloud" servers (den Bakker et al., 2014, Gardy et al., 2015, Quick et al., 2014). Portable near-future sequencing instruments will be at low cost to use and thus make it possible to deploy these instruments widely (Wang et al., 2015). The diagnostic tools used today to detect pathogens are developing towards portable sequencing platforms and bioinformatic methods by which one can identify and characterize any pathogen rapidly and reliably.

6. CONCLUDING REMARKS AND FUTURE PROSPECTS

In this project, the aim was to investigate whether rapid and reliable diagnosis of pathogenic agents with portable thermocyclers and pathogen-specific assays is realistic in biothreat preparedness and response.

The key findings were that modern technology combined with in-house pathogen-specific assays can yield DNA amplification data of a similar quality in field or laboratory situations. Sample preparation and pathogen identification methods used in this study proved to be rapid and reliable when performed in field conditions. Available hardware and optimized protocols can satisfy all the main tasks of the Finnish Defence Forces, namely military defense of Finland, support other domestic authorities, and to participate in international crisis management. Rapid diagnostics can be used to assist decision making in outbreak investigations (Figure 17).

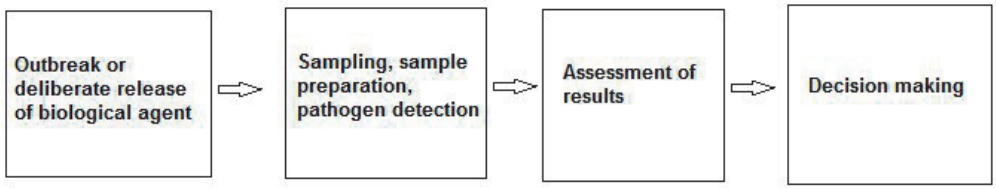


Figure 17. Rapid sample preparation and detection of a pathogen will hasten and improve decision making in outbreak situations or when investigating alleged deliberate release of biological agent.

Reduction in size and weight of near-future equipment for molecular detection of pathogens will help develop lab-in-a-box systems to a backpack laboratory (Inglis, 2013). Furthermore, increasingly user-friendly operating systems will reduce the need for highly trained personnel to safely obtain diagnostic data at short notice (Zumla et al., 2014).

The boundary between laboratory and field analysis is blurring as novel detection and sequencing technologies are developing and becoming more suitable for use outside the laboratory (Bertelli and Greub, 2013). Discrimination between deliberate release of a biological agent and its natural cycle requires detailed characterization of the agent. With accurate technology and a complete knowledge-base, a modular field laboratory can provide advanced technical support in resource-limited and remote locations where clinical laboratories are unavailable (Inglis, 2013). The present study provides methods

that can be used in a field laboratory to be deployed at short notice. This system provides authorities with a rapid-response capability to biothreats. A robust and accountable protocol provides excellent documentation for decision-makers and agencies wishing to pursue legal action with respect to any wrongdoing. The ability to inform public health decision makers with rapid diagnostics has proven useful elsewhere (Inglis et al., 2011).

This study has identified several issues that require additional work. First, there is a need to define what "identification" actually means in the context of a biological weapon investigation, and the interpretation of results must be very carefully defined (II, III, IV). Key factors will be precise and robust procedures that must be followed in any protocol, and certification and accreditation processes must focus on general performance (Bonini et al., 2002).

Another important issue in biological threat preparedness is the systematic risk assessment based on scenarios, both current and those yet to be realized. The rapidly changing world will present new opportunities to hostile agencies wishing to cause harm, and social media platforms provide ample opportunities for acquiring and deliberately disseminating false information regarding biothreats. Information spreads rapidly and creates general feelings of insecurity among the general public.

In this study, experience gained during field trials in Finland and Tanzania show that diagnostic work outside the laboratory can face many challenging situations e.g., limited and compromised workspace, extreme temperatures, and an unstable power supply. These situations can be overcome with available technology and rapid, reliable field diagnostic systems are realistic and within reach. It is expected that lightweight and highly portable "backpack laboratories" will soon become available that are battery powered and rely on simple robust protocols that yield easily-interpreted results. Wireless communication technology will enable the rapid delivery of information to decision makers and other parties. Such diagnostic technologies offer a reliable solution to localized and rapid identification of biological agents in outbreaks or investigation programs.

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Markos Mölsä
Helsinki, November 2016

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9. ORIGINAL PUBLICATIONS

ARTICLE I. Markos Mölsä, Laura Kalin-Mänttäri, Elina Tonteri, Heidi Hemmilä, Simo Nikkari (2016). Comparison of four commercial DNA extraction kits for the recovery of Bacillus spp. spore DNA from spiked powder samples. J Microbiol Methods. 2016 Sep;128:69-73.

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ARTICLE III. Markos Mölsä, Katja Koskela, Esa Rönkkö, Niina Ikonen, Thedi Ziegler, Simo Nikkari (2012). Detection of influenza A viruses with a portable real-time PCR instrument. J Virol Methods. 2012 May;181(2):188-91

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Comparison of four commercial DNA extraction kits for the recovery of *Bacillus* spp. spore DNA from spiked powder samples

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ABSTRACT

Bacillus spp. include human pathogens such as *Bacillus anthracis*, the causative agent of anthrax and a biothreat agent. *Bacillus* spp. form spores that are physically highly resistant and may remain active over sample handling. We tested four commercial DNA extraction kits (QIAamp DNA Mini Kit, RTP Pathogen Kit, ZR Fungal/Bacterial DNA MiniPrep, and genesig Easy DNA/RNA Extraction kit) for sample inactivation and DNA recovery from two powders (icing sugar and potato flour) spiked with *Bacillus thuringiensis* spores. The DNA was analysed using a *B. thuringiensis*-specific real-time PCR assay. The detection limit was 3×10^1 CFU of spiked *B. thuringiensis* spores with the QIAamp DNA Mini, RTP Pathogen, and genesig Easy DNA/RNA Extraction kits, and 3×10^3 CFU with the ZR Fungal/Bacterial DNA MiniPrep kit. The results showed that manual extraction kits are effective and safe for fast and easy DNA extraction from powder samples even in field conditions. Adding a DNA filtration step to the extraction protocol ensures the removal of *Bacillus* spp. spores from DNA samples without affecting sensitivity.

1. Introduction

Rapid and accurate molecular detection and diagnosis of infectious agents is crucial in preparedness for infectious diseases and biothreats (Inglis, 2015; Zasada et al., 2015; Coyne et al., 2004). Inactivation and nucleic acid extraction are often laborious and time-consuming steps needed before DNA analysis. Especially when working in field conditions sample preparation is challenging and a potential cause for inaccurate diagnostic results (Bonini et al., 2002; Panning et al., 2007; Lermen et al., 2014; Wolfel et al., 2015). Safe DNA analysis is challenging if the sample contains pathogenic bacterial spores that have remained active after extraction with a commercial extraction kit (Dauphin and Bowen, 2009; Panning et al., 2007; Schmidt et al., 2011).

Bacillus spp. includes human pathogens such as *Bacillus anthracis*, the causative agent of anthrax (Schmidt et al., 2011). These Gram-positive spore-forming bacteria are found in soil and are mainly associated with animals. Human anthrax spreads usually through infected livestock when handling infected animals (Goel, 2015). *B. anthracis* spores enter the body either through a skin lesion (cutaneous anthrax), lungs (pulmonary anthrax), or gastrointestinal route (gastrointestinal anthrax) (Anderson and Bokor, 2012). Anthrax is a public health concern in countries where agriculture is an important source of income and where widespread vaccination of animals is not practiced (Goel, 2015). Because of the extremely resistant spores and high mortality rates, *B. anthracis* is also a potential biothreat agent (Anderson and Bokor, 2012), and both accidental and deliberate releases have been reported (Jernigan et al., 2001; U.S Department of Defence, 2015) (Jackson et al., 1998). Another spore forming bacteria of the *Bacillus* genus, *Bacillus thuringiensis*, is widely used in pesticides as it has an ability to produce toxins, which are toxic to many insect pests (Arteaga et al., 2014). *B. thuringiensis* is not generally considered a human pathogen (Kaminska et al., 2014), thus it is a suitable simulant for isolation and extraction experiments with *Bacillus* genus spores (Janse et al., 2010b; Carrera et al., 2007).

Manual nucleic acid extraction are in routine use to date (Ip et al., 2015; Dauphin et al., 2011; Dauphin et al., 2010; Whitehouse and Hottel, 2007). Contrary to the previous studies, this study has an emphasis on comparison of DNA extraction kits for the recovery of *Bacillus* spp. spore DNA from spiked powder samples. Furthermore, the compared kits are employing different techniques, including heat treatment, spin column procedures, bead beating, and magnetic beads, and the study focuses on applying these techniques in field.

We compared the safety and efficiency of four commercial DNA extraction kits (QIAamp DNA Mini Kit, RTP Pathogen Kit, ZR Fungal/Bacterial DNA MiniPrep, and genesig Easy DNA/RNA Extraction kit). Swift and simple methods that can be used in field conditions were preferred. The ability of the kits to inactivate *B. thuringiensis* spores and the amount of extracted DNA from spiked powder samples (icing sugar and potato flour) were evaluated. The performance of the kits was evaluated

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based on the processing time, DNA yield and purity, the need for additional laboratory equipment (e.g. heat-block, vortex), the elimination of potential PCR inhibitors, and the sensitivity of subsequent real-time PCR analysis. Also centrifugal filter units (Millipore Ultrafree-MC Centrifugal Filter Devices) were tested for the removal of spores from DNA samples.

2. Materials and methods

2.1. Spiking and preparation of powder samples.

Icing sugar and potato flour were spiked with an insecticidal product (TUREX 50 WP, Certis, Columbia, MD, USA), containing *B. thuringiensis* ssp. *kurstaki-aizaway* strain GC-91 spores. A spiking stock of TUREX powder (4 g) and nuclease-free water (NFW) (Sigma-Aldrich, St. Louis, MO, USA) (100 ml) was prepared. The suspension was mixed by vortexing and divided into 1 ml aliquots and immediately stored at −70 °C. Three aliquots were thawed and diluted ten-fold with NFW to determine the colony forming unit (CFU) count of the spiking stock. 100 μl of dilutions from 10^{-6} to 10^{-12} were plated on two parallel lysogeny broth (LB) (Bertani, 2004) plates and incubated at 37 °C for 21 h. CFU count was calculated based on the number of visible colonies. Spiking was performed as follows: One aliquot of spiking stock (4% TUREX suspension) was freshly thawed and serially diluted 100-fold with NFW. Next, one part of spiking stock dilutions (10^{-2}, 10^{-4}, or 10^{-6}) were added as duplicates to four parts of 10% powder suspensions.

To control the homogeneity of the powder samples, they were handled as liquid suspensions. Thus, powder samples were prepared freshly by mixing 1 g of icing sugar or potato flour with 10 ml of NFW. Six parallel powder samples were spiked with TUREX dilutions as described above. In addition, three control samples were included in each DNA extraction assay: undiluted spiking stock as positive control and unspiked powder suspension as well as NFW as negative controls. All DNA extraction assays were performed with a total sample volume of 200 μl.

2.2. DNA extraction methods

DNA was extracted from a sample volume of 200 μl according to manufacturers' protocols for difficult-to-lyse or Gram-positive bacteria, if available. Elution volume was 100 μl. DNA samples were stored at −70 °C. When using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), DNA was extracted according to manufacturer's protocol "Isolation of genomic DNA from Gram-positive bacteria". With RTP Pathogen Kit (Stratec, Birkenfeld, Germany), the samples were prepared according to manufacturer's protocol, thus adjusted from 200 μl sample volume to a total volume of 400 μl using NFW, and DNA was extracted following the protocol "Extraction of DNA from bacterial pellets" for Gram-positive bacteria. DNA extraction using the ZR Fungal/Bacterial DNA MiniPrep (Zymo Research, Irvine, CA, USA) kit was performed according to the manufacturer's protocol. No beta-mercaptoethanol was added to the Fungal/Bacterial DNA Binding Buffer. For the Genesig Easy DNA/RNA Extraction kit (Primerdesign, Rownhams, Southampton, UK), the samples were prepared according to manufacturer's protocol for faeces/soil. DNA was extracted following the detailed protocol and using the Primerdesign Magnetic Rack.

2.3. Real-time PCR analysis

10-fold serial dilutions were made of extracted DNA ranging from 10^{-1} to 10^{-4} dilutions. All analyses were run as duplicates and amplification of the targeted template area in both parallel reactions was qualified as a positive result. *B. thuringiensis* ssp. *kurstaki-aizaway* strain GC-91 DNA was included in each PCR run as external positive control and no-template-control as negative control.

DNA was detected with *B. thuringiensis*–specific primers and probe targeting the *cry* gene, as described earlier (Matero et al., 2011). The amplification mixture in a total volume of 10 μl or 20 μl contained DyNAmo ColorFlash qPCR Mix (Thermo Scientific, Waltham, MA, USA), 0.3 μM of forward primer, 0.9 μM of reverse primer, 0.25 μM of probe, 0.01 U/μl AmpErase Uracil N-Glycosylase (Applied Biosystems, Carlsbad, CA, USA) for inhibition of PCR carryover contamination, the TaqMan Exo IPC Mix and Exo IPC DNA (Applied Biosystems) as internal positive control assay, template, and NFW. PCR reactions were performed using PikoReal Real-Time PCR System (Thermo Scientific). Reaction conditions were 2 min at 50 °C, 7 min at 95 °C, followed by 45 cycles of 5 s at 95 °C and 30 s at 60 °C.

2.4. Measuring DNA concentration and purity

The NanoDrop™ One Spectrophotometer (Thermo Scientific, Software version 1.1.0) was used for measuring of DNA concentration and purity after DNA extraction. The related elution buffer was used as a blank and DNA measured from two parallel 1 μl samples per eluate.

2.5. Evaluation of bacterial spore inactivation or removal

B. thuringiensis spore inactivation was studied by incubating 10% of the extracted DNA sample volume (i.e. 10 μl) originating from positive extraction controls on three parallel nutrient-rich LB plates at 37 °C for 14 days. Positive extraction control DNA was chosen for plating as the original sample (undiluted spiking stock) contained the highest spore amount compared to the other samples, and thus it would challenge the kits' inactivation capacity the most. Freshly thawed *B. thuringiensis* spiking stock served as a positive and elution buffers from each kit as negative plating controls.

2.6. DNA filtration

Centrifugal filtering was used for removal of spores from the DNA samples originating from the positive extraction controls. 40 μl of the extracted DNA was filtered with Millipore Ultrafree-MC Centrifugal Filter Devices with a pore size of 0.1 μm (Merck, Kenilworth, NJ, USA) by centrifugation at 12.000 × *g* for 4 min. Freshly thawed *B. thuringiensis* spiking stock and DNA elution buffer in question were used as controls. The removal of the spores was verified by culturing as described above.

3. Results

A simplified workflow from sample preparation to DNA extraction and subsequent analyses is presented in Fig. 1.

Depending on the kit, the processing time of nine samples varied from 65 min to 155 min and DNA yield from 17 to 78 ng/μl (Table 1). Potato flour caused clogging of the RTP Pathogen Kit's spin filters and reduced the total volume of eluted DNA from 100 μl to approximately 45 μl (data not shown). Additional laboratory equipment was needed with all four kits (Table 1).

The kits' efficiency to extract spore DNA from spiked powder samples was determined with a *B. thuringiensis*–specific real-time PCR assay. The detection limit was 3×10^1 CFU of spiked spores with the QIAamp DNA Mini, RTP Pathogen and genesig Easy DNA/RNA Extraction kits, and 3×10^3 CFU with the ZR Fungal/Bacterial DNA MiniPrep kit (Fig. 2). Internal positive control (IPC) assay was included in all real-time PCR reactions for detection of possible PCR inhibitors. No signs of PCR inhibitors were noticed.

To evaluate the sample inactivation efficiency of the extraction protocols, DNA aliquots originating from positive extraction controls were incubated on LB plates. DNA extracted with the genesig Easy DNA/RNA Extraction kit showed bacterial growth after 18 h of incubation (Table 1). DNA extracted using the other three kits did not show any bacterial growth in 14 days, after which the test was terminated. DNA

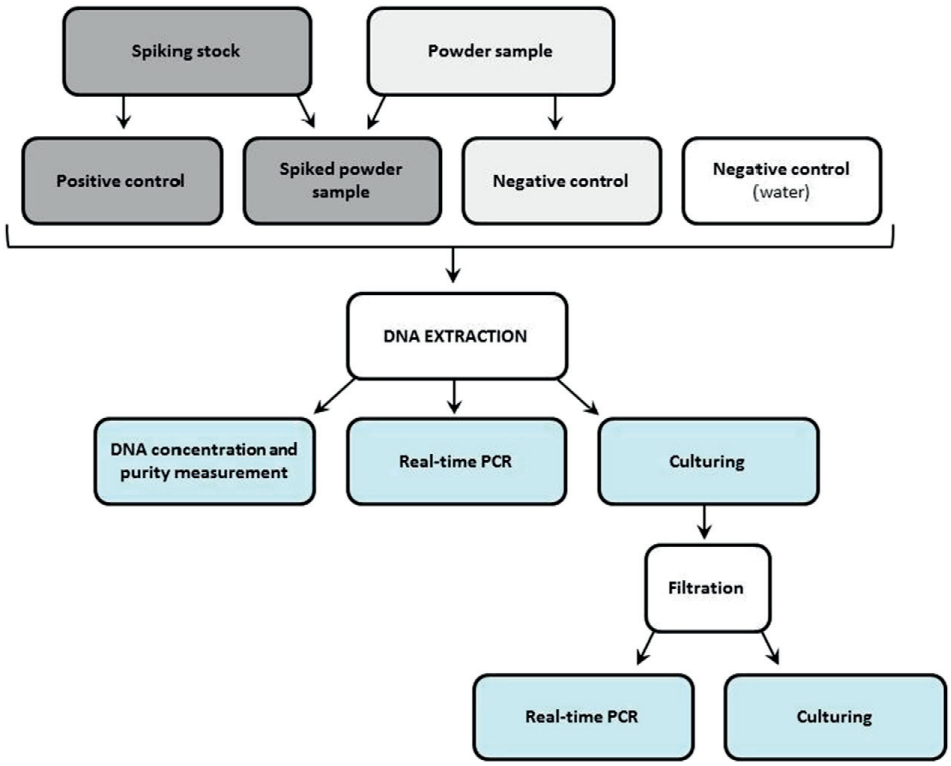

Fig. 1. A workflow of the study from sample preparation to DNA extraction and subsequent analyses: First, powder samples and extraction controls were prepared. Next, DNA extractions were performed according to manufacturers' protocols for difficult-to-lyse or Gram-positive bacteria. Extracted DNA was evaluated by measuring the concentration and purity, and by a real-time PCR analysis. Bacterial spore inactivation was studied by culturing DNA originating from positive extraction controls. Finally, centrifugal filtering was used for removal of spores from the DNA samples, and verified by culturing and real-time PCR.

recovered from the genesig Easy DNA/RNA Extraction kit was filtered using Millipore filters, after which no viable spores were detected by bacterial culture. According to the real-time PCR results, filtration did not affect the sensitivity of the method.

Table 1
Main features and performance indicators of the DNA extraction kits.

		DNA extraction kit			
		QIAamp DNA Mini Kit	RTP Pathogen Kit	ZR Fungal/Bacterial DNA MiniPrep	genesig Easy DNA/RNA Extraction kit
Features	Method	Spin column	Spin column	Spin column	Magnetic beads
	Extra equipment[a]	2 heat blocks	Heat block, thermomixer	–	Magnetic rack
	Lysis method	Heat treatment	Heat treatment	Lysis solution + bead beating	Lysis buffer
	Cost/sample (USD)[b]	4.5	6.2	3.3	5.7
Performance indicators	Spore inactivation[c]	Yes	Yes	Yes	No
	Processing time[d]	155 min	100 min	65 min	105 min
	DNA yield[e]	34 ng/μl	78 ng/μl[f]	17 ng/μl	41 ng/μl
	DNA purity (A260/A280)[e]	1.8	2.5	1.5	1.4
	Ct value[g]	18.4	18.7	19.0	19.4
	Limit of detection[h]	3×10^1 CFU	3×10^1 CFU	3×10^3 CFU	3×10^1 CFU

[a] In addition to a vortex and a minispin.
[b] Kits for 50 preparations.
[c] Without additional lysis or DNA filtration methods.
[d] With nine samples.
[e] Average of two parallel measurements/reactions of positive extraction control samples.
[f] Includes carrier-RNA.
[g] *B. thuringiensis*-specific real-time PCR assay.
[h] Limit of detection of the extracted DNA by *B. thuringiensis*-specific real-time PCR assay; CFU of spiked spores.

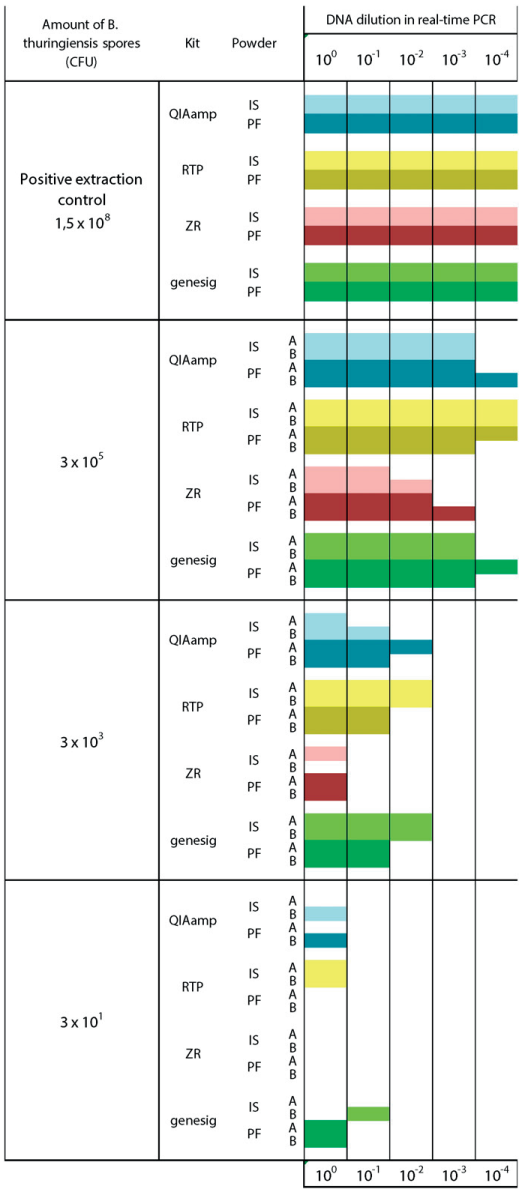

Fig. 2. The recovery of *B. thuringiensis* spore DNA from spiked powder samples with four extraction kits evaluated by *B. thuringiensis*-specific real-time PCR analysis. QIAamp = QIAamp DNA Mini Kit; RTP = RTP Pathogen Kit; ZR = ZRFungal/Bacterial DNA MiniPrep; genesig = genesig Easy DNA/RNA Extraction kit; IS = icing sugar; PF = potato flour; A and B = duplicate samples.

4. Discussion

All tested extraction kits were considered easy to use, thus potentially suitable for field conditions. The compared extraction kits employed different techniques, including heat treatment, spin column procedures, bead beating, and magnetic beads (Table 1). These techniques had an effect on the processing time (Table 1) and the performance of the kits, which was seen as sensitivity differences in real-time PCR results (Fig. 1). When following the protocols provided by the manufacturers, three of the four kits inactivated *B. thuringiensis* spores.

The QIAamp DNA Mini Kit and RTP Pathogen Kit protocols included 10–15 min heat incubation steps at 95 °C and thus required heating blocks as extra equipment in addition to a vortex and a centrifuge. Heating is effective for sample inactivation and for elimination of potential PCR inhibitors (Zhang et al., 2010) but is often also the most time-consuming step. Processing time with the QIAamp DNA Mini Kit was the slowest with 155 min and DNA yield the second lowest with 34 ng/μl (Table 1). However, the QIAamp DNA Mini Kit resulted the highest purity, with a A260/A280 ratio of 1.8 (Table 1).

The extraction protocol of the RTP Pathogen Kit took 100 min and – according to NanoDrop – it yielded the most DNA (Table 1). However, the RTP Pathogen Kit is designed for simultaneous extraction of total nucleic acids, which in general is an advantage in field use, but affects the absorbance reading as the sample will contain also RNA. In addition, carrier-RNA is included in the extraction reagents for the enhancement of viral DNA/RNA recovery as well as for stabilization of nucleic acids in samples. Therefore eluates will contain carrier-RNA, which – according to the manufacturer – will greatly exceed the amount of the isolated nucleic acids. The manual instructs that quantification of DNA and RNA must be done by means of amplification or hybridization-based measurements. Other methods, such as absorption measurements will be disturbed by the included carrier-RNA as well as DNA or RNA which is co-purified. This is also evident in this study, as the nucleic acid yield was exceptional with 78 ng/μl with a high A260/A280 ratio value of 2.5 (Table 1). Variations in total volume of eluted DNA between the two powders indicate that the RTP Pathogen Kit's spin filters were clogged by potato flour, and can be clogged by powder samples of similar consistency. Similar occurrence was not noticed with icing sugar. The results suggest that this difference affects the DNA yield and consequently the sensitivity of the real-time PCR (Fig. 2).

The ZR Fungal/Bacterial DNA MiniPrep protocol offered the fastest extraction, 65 min, and the only extra equipment needed to complete the protocol were a vortex and a mini spin. Yet the kit's performance was the weakest with a DNA yield of 17 ng/μl (Table 1) and according to the sensitivity of the real-time PCR analysis (Fig. 2). However, ZR Fungal/Bacterial DNA MiniPrep kit has been previously used for screening of *Francisella tularensis* from vole liver samples in the field, which shows that the performance level of such is valid for pathogen detection from tissues (Molsa et al., 2015). Furthermore, in the previous studies higher DNA yields have been obtained using bead mill homogenization (Miller et al., 1999). All three kits (QIAamp DNA Mini Kit, RTP Pathogen Kit and ZR Fungal/Bacterial DNA MiniPrep kit) inactivated *B. thuringiensis* spores and spore DNA could be extracted from spiked powder samples.

Extraction process with the genesig Easy DNA/RNA Extraction kit took 105 min and DNA yield was 41 ng/μl (Table 1). The genesig Easy DNA/RNA Extraction kit method requires only a magnetic rack as extra equipment, which makes it a promising candidate for field use where no electricity is available. However, bacterial growth occurred on LB plates after the DNA extraction, i.e. the kit did not inactivate *Bacillus* spores in the samples. DNA extraction with the genesig Easy DNA/RNA Extraction kit was repeated five times and the recovery of viable *B. thuringiensis* was seen every time. According to the kit's manual, sample inactivation is achieved chemically by incubation with a lysis buffer containing chaotropic ions supported by proteinase K digestion and mechanical disruption or treatment using suitable glass beads is recommended for bacteria difficult to lyse. This step would require extra equipment and time, and was therefore not done in this study aiming to quick and easy DNA extraction. The other tested kits that inactivated the spores completely, include either incubation periods of 10–15 min

at 95 °C (QIAamp DNA Mini Kit, RTP Pathogen Kit) or samples are lysed mechanically by bead beating (ZR Fungal/Bacterial DNA MiniPrep) in addition to a lysis solution.

Icing sugar and potato flour samples were spiked with an insecticidal product (TUREX 50 WP) containing spores of *B. thuringiensis*. The spores have physical dimensions (length 2 μm, width 1 μm) similar to *B. anthracis* (length 1.07 μm, diameter 0.48 μm) (Carrera et al., 2007). This makes *B. thuringiensis* a usable spore simulant for *B. anthracis* for DNA extraction and centrifugal filter experiments (Janse et al., 2010a). The selected centrifugal filter units have a filter pore size of 0.1 μm, which has been previously reported to remove spores successfully from samples (Dauphin and Bowen, 2009). Also in this study the centrifugal filter units removed the spores both from bacterial spore positive DNA samples extracted with the genesig Easy DNA/RNA Extraction kit and from bacterial spore positive control samples. Considering the genesig Easy DNA/RNA Extraction kit's fast extraction time with a low amount of equipment, the kit used together with the centrifugal filters seems highly usable in field conditions.

The real-time PCR results reflected the DNA yields: The detection limits of the three extraction kits with higher DNA yield (QIAamp DNA Mini Kit, RTP Pathogen Kit, genesig Easy DNA/RNA Extraction kit) were 3×10^1 CFU of spiked *B. thuringiensis* spores, or one grade better than with the ZR Fungal/Bacterial DNA MiniPrep kit (Fig. 2, Table 1). Overall, all four kits gave consistent results in real-time PCR. The difference in cost per sample between the four tested kits was significant but the results indicated no clear correlation between the overall performance and the price (Fig. 2, Table 1).

Possible impurities and/or flour DNA did not seem to interfere with detection of *B. thuringiensis*–specific DNA. No cross-contamination of negative control samples was noticed with any of the tested extraction kits. Cross-contamination is a major concern, especially with automated extraction systems (Smith et al., 2003; Knepp et al., 2003). Automated DNA extraction methods can result in better real-time PCR detection levels than manual extraction kits, as factors like DNA purity and concentration can influence the sensitivity of real-time PCR assays (Dauphin et al., 2011). Light sample preparation methods with a small footprint, like the tested manual extraction kits in this study, may be more suitable in field, e.g. used with foldable glovebox systems. A glovebox system would also increase the biosafety of sample handling in addition to personal protective equipment (PPE) (Wolfel et al., 2015; Grolla et al., 2012; Panning et al., 2007).

The aim of this study was to compare the performance of four commercially available DNA extraction methods for their ability to inactivate bacterial spores and to extract spore DNA from spiked flour samples. The results demonstrate that the manual extraction kits added with centrifugal filters are suitable for safe, fast, and easy extraction of bacterial DNA from powder samples in a sophisticated laboratory environment as well as in field conditions.

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Monitoring biothreat agents (*Francisella tularensis*, *Bacillus anthracis* and *Yersinia pestis*) with a portable real-time PCR instrument

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ABSTRACT

In the event of suspected releases or natural outbreaks of contagious pathogens, rapid identification of the infectious agent is essential for appropriate medical intervention and disease containment. The purpose of this study was to compare the performance of a novel portable real-time PCR thermocycler, PikoReal™, to the standard real-time PCR thermocycler, Applied Biosystems® 7300 (ABI 7300), for the detection of three high-risk biothreat bacterial pathogens: *Francisella tularensis*, *Bacillus anthracis* and *Yersinia pestis*. In addition, a novel confirmatory real-time PCR assay for the detection of *F. tularensis* is presented and validated. The results show that sensitivity of the assays, based on a dilution series, for the three infectious agents ranged from 1 to 100 fg of target DNA with both instruments. No cross-reactivity was revealed in specificity testing. Duration of the assays with the PikoReal and ABI 7300 systems were 50 and 100 min, respectively. In field testing for *F. tularensis*, results were obtained with the PikoReal system in 95 min, as the pre-PCR preparation, including DNA extraction, required an additional 45 min. We conclude that the PikoReal system enables highly sensitive and rapid on-site detection of biothreat agents under field conditions, and may be a more efficient alternative to conventional diagnostic methods.

1. Introduction

The Centers for Disease Control and Prevention (CDC) lists three bacterial species as category A biological threat agents: *Francisella tularensis*, *Bacillus anthracis* and *Yersinia pestis* — which cause the human diseases tularemia, anthrax and plague, respectively (Centers for Disease Control and Prevention (CDC), 2015). These zoonotic agents are highly pathogenic, causing potentially high case fatality rates, and are a major risk to public health (Anderson and Bokor, 2012). Rapid identification and accurate characterization are essential for appropriate control measures, as timely post-exposure antibiotic treatment can usually protect against symptomatic infections (Ivnitski et al., 2003; Irenge and Gala, 2012). The risks of these bacteria are compounded by their ability to circulate as unnoticeable aerosols (Katz and Zilinskas, 2011), and an incubation period of up to multiple weeks following human exposure (Anderson and Bokor, 2012; Koskela and Salminen, 1985; Dennis et al., 2001).

Several different diagnostic methods are available for the detection of bacterial infections, e.g., staining, serology and immune assay diagnostics (Grunow et al., 2000). However, relatively fast diagnostic tests, such as immune assay-based rapid tests, have limited sensitivity (Irenge and Gala, 2012), while others, such as conventional cultures, can take several days to obtain results (Grunow et al., 2000; Hatchette et al., 2009). Real-time PCR is well suited for the diagnostics of dangerous bacterial agents, due to its high sensitivity and specificity, and the possibility to inactivate samples before analysis (Grunow et al., 2000; Hatchette et al., 2009; Pohanka and Skladal, 2009). However, robust and reliable PCR equipment for the detection and characterization of pathogens is often large and heavy, and the analysis process may require several hours. Therefore, portable real-time PCR detection equipment with high specificity and sensitivity is urgently required.

The purpose of this study was to evaluate and further develop rapid and reliable field diagnosis technologies for multiplex PCR purposes. Two platforms, PikoReal (Thermo Fisher Scientific, Waltham, MA, USA), and ABI 7300 (Life technologies Ltd, Carlsbad, CA, USA), were compared for the real-time PCR detection of *F. tularensis*, *B. anthracis* and *Y. pestis*. Furthermore, a 23 kDa PikoReal assay for the detection of *F. tularensis* under field conditions was developed and validated. We

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demonstrated that highly accurate results can be achieved with this technology, with sensitivity and specificity comparable to established methods used in specialized diagnostic laboratories.

2. Materials and methods

2.1. Description of real-time PCR thermocyclers

The PikoReal system weighs 10 kg and is equipped with five LED illuminated optical channels, which increases durability when compared to standard light technologies, facilitates multiplexing, and enables the use of internal positive controls (Thermo Fisher Scientific Inc., 2015). The ABI 7300 is a 29 kg instrument with tungsten-halogen lamp technology (Koskela et al., 2009). It is commonly used for real-time PCR in diagnostic laboratories (Espy et al., 2006), and therefore serves as an appropriate comparison to evaluate the PikoReal system.

2.2. Oligonucleotides for PCR

The bacterial strains used to evaluate sensitivity and specificity of the real-time PCR assays are listed in Table 1 (Skottman et al., 2007; Matero et al., 2011). Five different PCR assays were compared using the two real-time PCR instruments (PikoReal and ABI 7300) (The oligonucleotides targeted the 23 kDa and ISFtu2 genes of *F. tularensis*, *cap* and *pag* genes of *B. anthracis*, and the *pla* gene of *Y. pestis*).

We created a novel assay for the detection of *F. tularensis*, targeting the ISFtu2 gene. The other oligonucleotides used in this study have been reported previously (Table 2) (Skottman et al., 2007). The ISFtu2 confirmatory assay targets the IS-element sequence of *F. tularensis*, which presents highly in the *F. tularensis* genome (Rohmer et al., 2007). The designed primer and MGB-probe (Life Technology Ltd, Carlsbad, CA, USA) combinations were based on sequences available from the public NCBI database (Accession number AY062040). The ISFtu2 confirmatory assay was developed using the Primer Express software, version 2.0 (Life Technologies Ltd, Carlsbad, CA, USA). 6-carboxy-fluorescein (FAM) was used as the fluorescent reporter dye at the 5′ end of the probe.

Oligonucleotide concentrations for real-time PCR were optimized in a matrix, as described previously (Skottman et al., 2007). Each PCR assay included a negative no-template control (NTC) and a positive DNA-control.

2.3. Optimization of the real-time PCR protocol

Three different commercial real-time PCR kits were evaluated for field use, following real-time PCR analysis: the QuantiFast Probe PCR Kit (Qiagen, Hilden, Germany), the Premix Ex Taq-kit (Takara, Shiga, Japan) and the DyNAmo ColorFlash qPCR Kit (Fisher Scientific, Vantaa, Finland). The DyNAmo ColorFlash qPCR kit was selected because it presented the highest sensitivity based on threshold cycles (C_t) values and fluorescence levels. The C_t-value is the intersection between a PCR amplification fluorescence curve and a threshold line. This kit enables the use of Uracil-N-glycosylase (UNG) enzyme, which hydrolyses uracil-glycosidic bonds in DNA containing dUTP and degrades the DNA into small fragments. Therefore, contamination with previous real-time PCR reactions is unlikely. This method also minimizes pipetting errors by providing a multicolor pipetting tracking system. The master-mix contains a blue dye, and a separate sample buffer contains a yellow dye. The real-time PCR reaction mix containing both components is green.

DNA was extracted with the Zymo Research Fungal/Bacterial DNA Mini Prep kit (Zymo Research Corp., Orange, CA). The reagents do not require freezer or cold storage, and it is therefore possible to store and

Table 1
Real-time PCR results demonstrating specificities of the assays targeting 23 kDa and ISFtu2 genes of *F. tularensis*, *cap* and *pag* genes of *B. anthracis* and *pla*-gene of *Y. pestis*. (−) describes a negative result and (+) describes a positive result.

			Bacillus anthracis				*Francisella tularensis*		*Yersinia pestis*		*Francisella spp.*	
			(*pag*)		(*cap*)		(23 kDa)		(*pla*)		(ISFtu2)	
Species	Strain(s)	Source[1]	ABI 7300	PikoReal	ABI 7300	PikoReal	ABI 7300	PikoReal	ABI 7300	PikoReal	ABI 7300	PikoReal
Agrobacterium tumefaciens	C58C1/RP4	1	−	−	−	−	−	−	−	−	−	−
B. anthracis	ATCC 4229 (pXO1-/pXO2+)	2	−	−	+	+	−	−	−	−	−	−
B. anthracis	Sterne 7702 (pXO1+/pXO2-)	2	+	+	−	−	−	−	−	−	−	−
B. cereus	ELMI 21	2	−	−	−	−	−	−	−	−	−	−
B. licheniformis	ELMI 325	2	−	−	−	−	−	−	−	−	−	−
B. mycoides	ELMI 44	2	−	−	−	−	−	−	−	−	−	−
B. thuringiensis	ELMI 123	2	−	−	−	−	−	−	−	−	−	−
B. thuringiensis	Subsp. kurstaki-aizaway	3	−	−	−	−	−	−	−	−	−	−
Brucella melitensis	72, biotype 3	4	−	−	−	−	−	−	−	−	−	−
Enterobacter cloacae	tks461	1	−	−	−	−	−	−	−	−	−	−
Escheria coli	C600/pYET6	1	−	−	−	−	−	−	−	−	−	−
F. tularensis	LVS (ATCC 29684)	5	−	−	−	−	+	+	−	−	+	+
Moraxella catarrhalis	035E	1	−	−	−	−	−	−	−	−	−	−
Staphylococcus aureus	ATCC 25923	1	−	−	−	−	−	−	−	−	−	−
Y. bercovieri	3016/84	1	−	−	−	−	−	−	−	−	−	−
Y. enterocolitica	1309/80	1	−	−	−	−	−	−	−	−	−	−
Y. intermedia	9/85	1	−	−	−	−	−	−	−	−	−	−
Y. kristensenii	119/84	1	−	−	−	−	−	−	−	−	−	−
Y. mollaretii	92/84	1	−	−	−	−	−	−	−	−	−	−
Y. pestis	EV76-c	1	−	−	−	−	−	−	+	+	−	−
Y. pestis	KIM D1	1	−	−	−	−	−	−	+	+	−	−
Y. pseudotuberculosis	H305-36/89	1	−	−	−	−	−	−	−	−	−	−
Y. pseudotuberculosis	No.90	1	−	−	−	−	−	−	−	−	−	−
Y. ruckeri	RS41	1	−	−	−	−	−	−	−	−	−	−

[1]The sources are:
(1) Laboratory Strain collection, Department of Bacteriology and Immunology, University of Helsinki.
(2) Laboratory Strain Collection, Finnish Food Safety Authority Evira.
(3) Purified from a commercial insecticide.
(4) Institute of Microbiology, German Armed Forces, Munich, Germany.
(5) Laboratory Strain Collection, FOI, Umeå, Sweden.

Table 2
The oligonucleotides were designed and optimized in final concentration to target 23 kDa and ISFtu2 genes of *F. tularensis*, *cap* and *pag* genes of *B. anthracis* and the *pla* gene of *Y. pestis*.

Target gene	Primer and probe sequences	Final concentration nM		Amplicon length (bp)	References
		ABI 7300	PikoReal		
cap	Forward 5′-TTG GGA ACG TGT GGA TGA TTT-3′	300	900	69	Skottman et al. (2007)
	Reverse 5′-TCA GGG CGG CAA TTC ATA AT-3′	900	900		
	Probe 5′-FAM-TAG TAA TCT AGC TCC AAT TGT-MGBNFQ-3′	250	250		
pag	Forward 5′-CGG ATA GCG GCG GTT AAT C-3′	300	900	85	Skottman et al. (2007)
	Reverse 5′-CAA ATG CTA TTT TAA GGG CTT CTT TT-3′	900	900		
	Probe 5′-FAM-TAG AAA CGA CTA AAC CGG ATA T-MGBNFQ-3′	250	250		
23 kDa	Forward 5′-TGA GAT GAT AAC AAG ACA ACA GGT AAC A-3′	300	900	84	Skottman et al. (2007)
	Reverse 5′-GGA TGA GAT CCT ATA CAT GCA GTA GGA-3′	900	900		
	Probe 5′-FAM-CCA TTC ATG TGA GAA CTG -MGBNFQ-3′	250	250		
ISFtu2	Forward 5′-TGC TTG TGC TAC GGG ATA TGA TA-3′	300	300	98	This study
	Reverse 5′-CTA AAG CAT CAG TCA TAG CAT GGA TT-3′	900	900		
	Probe 5′-FAM- AGA TGA TAA CCA AGC AAT T-MGBNFQ-3′	250	250		
pla	Forward 5′-GAA AGG AGT GCG GGT AAT AGG TT-3′	50	300	63	Skottman et al. (2007)
	Reverse 5′-CCT GCA AGT CCA ATA TAT GGC ATA-3′	300	900		
	Probe 5′-FAM-TAA CCA GCG CTT TTC -MGBNFQ-3′	250	250		

use the kit at room temperature. These stable reagents and the short manual protocol enable isolation of DNA from one sample in 15 min. The DNA extraction method, including all required instruments, was packed into a portable case (Sniegel design, Sweden) for field deployment.

2.4. Sensitivity and specificity testing of the PCR assays

Sensitivity and specificity of the *F. tularensis*, *B. anthracis* and *Y. pestis* assays were each evaluated with the PikoReal and ABI 7300 real-time PCR systems. Sensitivity of the assays was tested using serial tenfold dilutions of DNA extracted from *F. tularensis LVS (ATCC29684)*, *B. anthracis* (Sterne 7702, pXO1+/pXO2- and ATCC 4229, pXO1-/pXO2+) and *Y. pestis* (EV76-c and KIM D1) strains. The specificity of the assays was evaluated with DNA extracted from 24 clinical and environmental bacterial species (Table 1).

Real-time PCR assays with both PCR platforms were performed in duplicate as part of a dilution series and each reaction was carried out with optimized oligo concentrations and a 2,5 μl DNA template in a final volume of 25 μl (Table 2). A no-template control (NTC) consisting of water instead of DNA as template, and a positive control containing bacterial DNA, was included in each assay. Real-time PCR assays with the PikoReal and ABI 7300 systems were performed using the following thermocycling parameters: 1 min at 60 °C and 2 min at 50 °C, 7 min at 95 °C, 45 cycles of 5 s each at 95 °C; 30 s at 60 °C, and 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s each at 95 °C respectively.

Data analysis was performed with the ABI 7300 software version 1.4 (ABI) and PikoReal SW-software version 2.0 (Thermo Fisher Scientific). Tests were performed at the diagnostic laboratory of the National Institute for Health and Welfare, Finland (THL).

2.5. Detection of F. tularensis using the PikoReal instrument under simulated field conditions

The PikoReal technology was also evaluated under field conditions. The PikoReal was packed into a compact, portable and shock and water resistant Pelican box (Pelican Products Inc., Torrance, CA), together with the entire hardware requisite for real-time PCR, e.g., a laptop with PikoReal software, and conductor rails for power supply. The DNA extraction equipment was transported in a separate case, as described above.

Rodent (voles and lemmings) liver samples were used to test for the presence of *F. tularensis*. These rodents were captured via snap (kill)-trapping at sites across Finland, dissected, and liver samples were collected and stored at −20 °C for subsequent DNA extraction. Liver has been previously demonstrated as an appropriate tissue for tularemia detection (Rossow et al., 2014a).

A total of 1035 samples were analyzed for *F. tularensis* (23 kDa) using the PikoReal assay at the Suonenjoki Research Station of the Finnish Forest Research Institute (now the Natural Resources Institute Finland) and the Kilpisjärvi Biological Station of the University of Helsinki. These facilities do not contain specialized diagnostic laboratories. From these samples 147 were also analyzed for *F. tularensis* (23 kDa) with the ABI 7300 system, including an internal positive control (IPC), as a confirmatory instrument platform in a well-equipped diagnostic laboratory at THL. The concentration and purity of the extracted DNA from the 147 samples was determined with the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Although all these rodent samples were negative to *F. tularensis*, they served to permit the large-scale utilization of the portable assay system outside of specialized diagnostic laboratories. These negative results are not surprising given the low and focal prevalence of *F. tularensis* in Finland (Splettstoesser et al., 2009; Rossow et al., 2014a), and rapid lethal clinical course of infection in rodents (Rossow et al., 2014b).

To further evaluate the process, five previously known *F. tularensis* positive vole liver samples (Rossow et al., 2014a) were extracted with the Zymo Research Fungal/Bacterial DNA Mini Prep kit (Zymo Research Corp., Orange, CA) and with the previously used reference extraction method, the Wizard®Genomic DNA Purification Kit (Promega, Madison, USA). Extracted DNA samples were analyzed with both the PikoReal and ABI 7300 systems at the THL laboratory.

2.6. Ethics and biosafety

All animal trapping took place with permission (1013/204/2002) on land owned by the Finnish Forest and Park Service. Permits (23/5713/2001, 4/5713/2007) for capturing protected species (here *Lemmus lemmus*) were granted by the Finnish Ministry of the Environment. Other species captured in this study are not protected in Finland and none of the captured species are included in the Red List of Endangered Species.

All animal tissue samples were inactivated with lysis buffer in the field prior to DNA extractions, and real-time PCR testing. Laboratory personal protective equipment (PPE) was used when handling tissue samples and working with the DNA-extraction protocol and real-time PCR. Following PCRs, remaining samples of extracted DNA and their tubes were disposed of into biohazardous waste bins, which were collected by a private contractor for incineration.

3. Results

The duration of all PCR assays with the PikoReal and ABI 7300 technologies were 50 and 100 min, respectively. The sensitivity of assays ranged from 1 to 100 fg of chromosomal *F. tularensis LVS*

Table 3
The detection limit of the assays ranged from 1 to 100 fg of chromosomal DNA. The highest sensitivity with PikoReal thermocycler and with ISFtu2 assay was obtained with 45 cycles.

Target bacteria	Sensitivity (limit of detection)[1]		
	Target gene	ABI 7300 (40 cycles)	PikoReal (45 cycles)
Bacillus anthracis	*cap*	100 fg	100 fg
	pag	10 fg	10 fg
Francisella tularensis	23 kDa	10 fg	10 fg
	ISFtu2	10 fg	1 fg
Yersinia pestis	*pla*	10 fg	10 fg

[1] Amount of genomic DNA based on dilution series.

(ATCC29684), *B. anthracis* (Sterne 7702, pXO1 +/pXO2- and ATCC 4229, pXO1-/pXO2 +) and *Y. pestis* (EV76-c and KIM D1) DNA, as determined by 10 parallel analyses with each platform (Table 3). Highest sensitivity of the PikoReal system was obtained with 45 cycles, as compared to 40 cycles with the ABI 7300 system.

The novel confirmatory ISFtu2 assay for *F. tularensis* presented similar sensitivity to the 23 kDa assay (Fig. 1). In addition, the ISFtu2 assay also detects *F. philomiragia*, which is not possible with the 23 kDa assay. No cross-reactivity was seen between species-specific assays or with DNA extracted from the 24 different other bacterial species used as templates (Table 1).

When analyzing rodent samples at the research stations in Suonenjoki and Kilpisjärvi, real-time PCR results using the PikoReal instrument were obtained in 95 min as the pre-PCR preparations, including DNA extraction, required approximately 45 min. Results from the PikoReal screening for *F. tularensis* at the research stations were in agreement with the 147 samples analyzed with both the ABI 7300 and PikoReal instruments in the diagnostic laboratory at THL. In all cases, the positive control was correctly identified. The five positive *F. tularensis* vole liver samples were accurately detected with both the PikoReal and ABI 7300 systems at the THL laboratory.

4. Discussion

A strong need exists for rapid, portable and reliable diagnostic methods for extremely contagious pathogens (Dennis et al., 2001; Grunow et al., 2012). Sensitivity and specificity of detection assays, as well as size and weight of the detection equipment, are the most important technical issues for the development of real-time PCR based assays for field use (Ivnitski et al., 2003). Due to suboptimal sensitivity with some of the available rapid immunoassay detection tests, nucleic acid based detection assays are becoming increasingly important to enable the fast and sensitive identification of harmful pathogens (Grunow et al., 2000; Hatchette et al., 2009; Irenge and Gala, 2012).

In this study, the field-deployable PikoReal system was used to develop a diagnostic assay for the reliable detection of selected biothreat bacterial agents. We found that this novel technology allows for rapid and sensitive detection of *F. tularensis*, *B. anthracis* and *Y. pestis*, and is suitable for utilization outside of sophisticated diagnostic laboratories. The PikoReal system demonstrated equal sensitivity and specificity when compared to a standard real-time PCR technology (ABI 7300).

Technical limitations often restrict the use of PCR diagnostics outside of sophisticated laboratories. One of the problems with many diagnostic PCR-based methodologies is the potential accumulation of amplified DNA fragments, which eventually lead to false positive results through contamination of sample materials and reagents (Burd, 2010). With the PikoReal assay the amplification occurs in a closed reaction plate. This effectively separates the amplified product from any part of the hardware and the environment. In addition, the selected real-time PCR methodology lowers pipetting and contamination risks when compared to PCR methods that require the reaction tubes to be opened during analysis.

Analyses with the PikoReal system proved to be simple and time-efficient. In our field trials, reliable results were achieved in approximately 90 min, from the beginning of sample preparation to the completion of the diagnostic PCR. These times are further enhanced by the lack of transportation required to deliver samples to specialized diagnostic laboratories. All assay-specific primers and probes, in both the PikoReal and the ABI 7300 systems, correctly identified all assay-specific positive controls while no cross-reactivity was observed.

Compared to earlier described portable PCR technologies (Koskela et al., 2009; Pierce et al., 2010; Matero et al., 2011; Molsa et al., 2012; Arif et al., 2013), the PikoReal system provides technical advances by facilitating multiplexing, and modules for absolute and relative quantification as well as melting curve analysis. These advances enhance the capabilities of the equipment for research use. The multicolor pipetting tracking system and possibility to use UNG-enzyme provided by the DyNAmo Color Flash qPCR kit, further increase the reliability of the assay. However, the performance of the field assays could be simplified by the development of stabilized or lyophilized real-time PCR reagents and assays with RT storage capability.

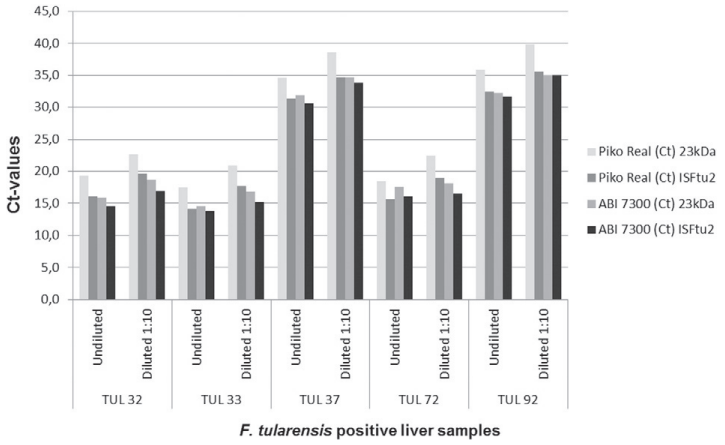

Fig. 1. The novel confirmatory ISFtu2 assay for *F. tularensis* achieved similar sensitivity to the 23 kDa assay. Results are based on five *F. tularensis* positive samples tested with each real-time PCR instrument (PikoReal and ABI 7300).

The PikoReal technology presents a strong candidate for further development to support more common public health diagnostic needs, such as respiratory disease-causing agents in primary health care settings. This lightweight and compact system allows rapid and sensitive detection of selected agents, and can be deployed outside of specialized diagnostic laboratories, rendering specimen transportation obsolete, and thereby greatly reducing time requirements and exposure risks.

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Conflicts of interest

The authors declare no competing conflicts of interest.

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Short communication

Detection of influenza A viruses with a portable real-time PCR instrument

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A B S T R A C T

Timely identification of respiratory pathogens is essential for appropriate patient care and cohorting. In order to do rapid identification-technology near the patient we utilized the field-deployable RAZOR EX-thermocycler with a reverse transcription real-time PCR assay that detects all subtypes of influenza A virus. In addition, we developed a RT PCR assay for specific detection of influenza A(H1N1)pdm09 virus. These assays amplified segments of the matrix (M)- and the hemagglutinin (HA)-gene, respectively. Detection limits of the M-gene and the influenza A(H1N1)pdm09-specific HA-gene assays were 0.15 PFU and 8.8 PFU per reaction, respectively. With 18 influenza A viruses of different subtypes and influenza B, C, and 7 other respiratory viruses the RAZOR EX and standard real-time PCR assay results were in total agreement. From 104 clinical samples identical results were obtained by both PCR methods. Additional 21 clinical samples were tested under field conditions with the RAZOR EX instrument. Results were achieved in 90 min, including 45 min for sample preparation and they were in complete agreement with those obtained by standard real-time PCR under laboratory conditions. These methods enable highly sensitive and rapid on-site diagnostics to reliably identify patients infected with influenza A, including the influenza A(H1N1)pdm09-virus.

The 2009 influenza A pandemic was a reminder that accurate diagnosis of respiratory tract infections remains a difficult task for the diagnostic laboratory, and although well-suited for outbreak investigations, available rapid point-of-care tests often lack sensitivity to identify single patients (Cheng et al., 2010; Ortiz de la Tabla et al., 2010). In the present study a field-compatible, portable PCR instrument was evaluated for the rapid, near-patient detection of influenza A viruses including A(H1N1)pdm09 in clinical specimens and compared with results obtained by a well-established real-time PCR instrument used under laboratory conditions.

Oligonucleotides for the specific detection of influenza A(H1N1)pdm09 were designed by aligning publicly available nucleotide sequences of segment 4 encoding the hemagglutinin (HA)-gene of influenza A viruses and used in reverse transcriptase (RT) polymerase chain reaction (PCR) assay (Table 1). In preliminary experiments (Rönkkö et al., 2011) the influenza A(H1N1)pdm09-specific assay described here proofed to be ten times more sensitive than the one published by CDC (Dawood et al., 2009). As a reference assay a published PCR targeting the matrix (M)-protein was used for broad-range detection of all subtypes of influenza A viruses (Munster et al., 2005; Ward et al., 2004) (Table 1). Testing of virus strains and clinical samples was performed with the RAZOR EX instrument (Idaho Technology, Salt Lake City, UT; software version 4.1), and results were compared to those obtained with the Applied Biosystems 7300 thermocycler (ABI 7300; ABI, Foster City, CA; software version 1.4). Both methods use Taqman chemistry. Initially, three different commercial RT PCR kits with five different protocols were evaluated for their performance by RT PCR. The One Step PrimeScript RT PCR Kit (Takara Bio Inc., Otsu, Japan) was selected for further use because highest test sensitivity was achieved repeatedly with this kit (data not shown). All PCR analyses were performed in duplicate according to the manufacturer's instructions. With the exception of the extension step, the thermal cycling profile was identical for the ABI and RAZOR methods and consisted of 5 min at 42 °C, 10 s at 95 °C, followed by 55 cycles of 5 s at 95 °C and 34 s or 20 s, respectively, at 60 °C. A no template control (NTC) consisting of water instead of RNA, and a positive control containing viral RNA were included in all PCR runs. The cell culture-grown influenza viruses A/Panama/2007/99(H3N2) or A/Finland/554/09(H1N1)pdm09 were included as positive controls in the M-protein and HA-gene assays, respectively, on both PCR methods.

The detection limits of the influenza A(H1N1)pdm09-assay and the broad-reacting influenza A type-specific assay were 8.8 PFU (plaque forming units) of cultured A/Finland/554/09 virus and as little as 0.15 PFU of cultured A/Panama/2007/99(H3N2) virus, respectively (data not shown). The specificity of the assays was tested with RNA from 18 influenza A viruses representing five different subtypes, the influenza A(H1N1)pdm09 virus, as well as with nine other common respiratory viruses obtained from the

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Table 1
Primers and probes as well as their optimal assay concentrations for type-specific and subtype-specific detection of influenza A and influenza A(H1N1)pdm09, respectively.

Target gene	Primer	Nucleotide sequence	Size of amplicon (bp)	Optimal concentrations (nM)
InfA matrix	Forward	5′-AAG ACC AAT CCT GTC ACC TCT GA-3′	95	900
	Reverse	5′-CAA AGC GTC TAC GCT GCA GTC C-3′		900
	Probe	5′-FAM-TTT GTG TTC ACG CTC ACC GTG CC-MGBNFQ-3′		250
InfA(H1N1)pdm09 hemagglutinin	Forward	5′-CAG GGA TGG TAG ATG GAT GGT AC-3′	122	900
	Reverse	5′-AAC AGA ATT TAC TTT GTT AGT AAT YTC GTC A-3′		900
	Probe	5′-FAM-CAG GAT ATG CAG CCG ACC TGA AGA GCA-BHQ-3′		250

National Influenza Centre (National Institute for Health and Welfare, Helsinki, Finland) (Table 2). With the M gene-specific reference assay, all influenza A-subtypes gave a positive signal while none of the other nine common respiratory viruses included in the control panel were amplified (Table 2). With the new assay described below, which is specific for the influenza A(H1N1)pdm09 virus, all the six pandemic influenza virus strains were positive (Table 2), while no reactivity was seen when other subtypes of influenza A or other common respiratory viruses were used as template. No difference in sensitivity or specificity between the ABI 7300 and the RAZOR EX-methods was observed.

RNA from 104 combined nasal and throat swabs was purified with the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) using the Qiacube instrument. These samples had been collected from military recruits presenting with symptoms of febrile upper respiratory tract infection during an outbreak in one garrison during the pandemic wave in the autumn of 2009 (Aho et al., 2010). One swab was used to collect material from both nostrils, the throat was sampled with the second swab. Both swabs were placed into the same tube containing 3 ml of transport medium (Copan UTM-RT, Copan Diagnostics Inc., Corona, CA). Of these 104 clinical samples, 65 were influenza A(H1N1)pdm09-positive and 39 negative, respectively, using both assays and PCR methods.

To demonstrate near-patient suitability of the RAZOR EX-technology, 21 clinical samples were tested under field conditions at a health care center of a military garrison Representative results from the analyses of these samples are shown in Fig. 1. RNA was extracted with the ZR Viral RNA Kit (Zymo Research, Orange, CA). After vortexing the clinical specimen for 1 min, an aliquot of 100 μl was transferred into a tube containing 300 μl of lysis buffer (ZR Viral RNA Buffer), and the extraction was performed according to manufacturer's protocol. In the final step the RNA was eluted in 35 μl of RNase-free water. RT PCR procedures with the RAZOR EX were performed as described above. With the RAZOR EX-instrument results could be obtained within 45 min from the beginning of PCR and an additional 45 min were required for the RNA extraction.

Optimally, laboratory confirmation of the disease-causing agent should be done near the patient in order to reduce time lost by specimen transportation. Unfortunately, some of the available point-of-care tests have suboptimal sensitivity (Ortiz de la Tabla et al., 2010) and cannot differentiate between pandemic and seasonal influenza viruses. On the other hand, technical constraints have thus far limited the use of PCR diagnosis near the patient, i.e., in a local health care center or a physician's office.

Portable thermocyler-technologies help to overcome these limitations. Recently, Wang et al. (2009) used the Smart Cycler instrument for identification of human and avian influenza A-viruses. However, the Smart Cycler is considerably larger and heavier (33.6 kg) than the compact and light weight (4.9 kg) RAZOR EX that is easily transportable by one person. Besides, the RAZOR EX performs thermocycling rapidly. In experiments carried out during this study reliable results were achieved in 1.5 h from the beginning of sample preparation to completion of the PCR, whereas for the Smart Cycler significantly longer period were required (Wang

Table 2
Analytical PCR results demonstrating specificities of the developed assays targeting the influenza A matrix gene and the hemagglutinin gene of influenza A(H1N1)pdm09 for type-specific influenza A (InfA) and subtype-specific influenza A(H1N1)pdm09 (InfA(H1N1pdm09)) detection, respectively. In addition 104 clinical samples were studied.

Virus	Source	InfA	InfA(H1N1)pdm09
Influenza A, A/Panama/2007/99 (H3N2)	Cultured virus	+	–
Influenza A, A/Fin/81/08 (H3N2)	Clinical sample	+	–
Influenza A, A/Fin/149/08 (H3N2)	Clinical sample	+	–
Influenza A, A/Fin/209/08 (H1N1)	Clinical sample	+	–
Influenza A, A/Fin/213/08 (H1N1)	Clinical sample	+	–
Influenza A, A/Mallard/Neth/12/00 (H7N3)	Cultured virus	+	–
Influenza A, A/HK/1073/99 (H9N2)	Cultured virus	+	–
Influenza A H5N1 Clade 1	Reference specimen[a]	+	–
Influenza A H5N1 Clade 2.1	Reference specimen[a]	+	–
Influenza A H5N1 Clade 2.2	Reference specimen[a]	+	–
Influenza A H5N1 Clade 2.3.2	Reference specimen[a]	+	–
Influenza A H5N1 Clade 2.3.4	Reference specimen[a]	+	–
Influenza A, A/Fin/544/09 (H1N1)pdm09	Clinical sample	+	+
Influenza A, A/Fin/571/09 (H1N1)pdm09	Clinical sample	+	+
Influenza A, A/Fin/577/09 (H1N1)pdm09	Clinical sample	+	+
Influenza A, A/Fin/579/09 (H1N1)pdm09	Clinical sample	+	+
Influenza A, A/Fin/582/09 (H1N1)pdm09	Clinical sample	+	+
Influenza A, A/Fin/554/09 (H1N1)pdm09	Cultured virus	+	+
Influenza B	Clinical sample	–	–
Influenza C, C/Ann Arbor/1/50	Cultured virus	–	–
Parainfluenza virus 1	Clinical sample	–	–
Parainfluenza virus 2	Clinical sample	–	–
Parainfluenza virus 3	Clinical sample	–	–
Respiratory syncytial virus	Clinical sample	–	–
Bocavirus	Clinical sample	–	–
Human metapneumovirus	Clinical sample	–	–
Adenovirus (type 7a)	Cultured virus	–	–

[a] Obtained through the WHO external quality assessment program. The A(H5N1) viruses represent 5 different clades and subclades

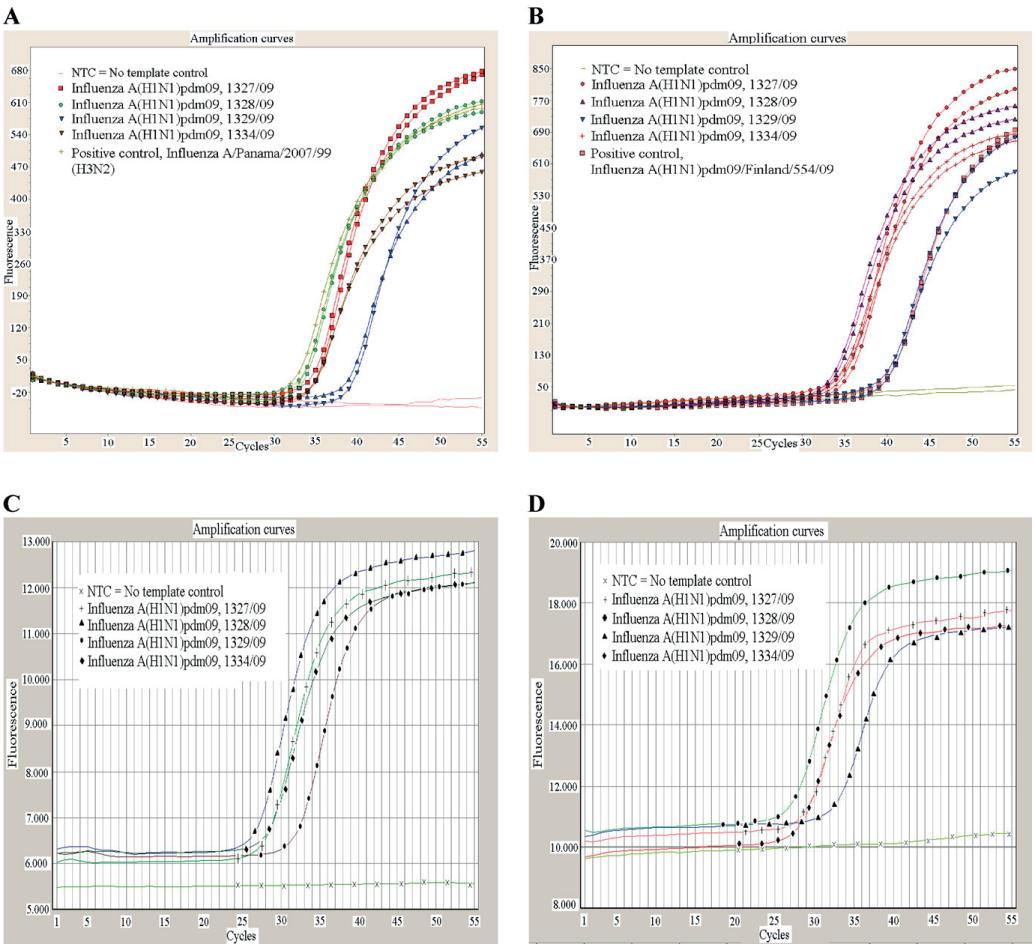

Fig. 1. Real-time RT PCR amplification of influenza A virus-specific gene segments with the RAZOR EX (A and B) and ABI 7300-instruments (C and D). RNA isolated from combined nasal and throat swabs of four patients with influenza A(H1N1)pdm09 infection was reverse transcribed and amplified by 55 cycles. Specimens were tested in duplicates by the RAZOR EX instrument. A specific product was amplified from all samples when influenza A type-specific (A and C) and influenza A(H1N1)pdm09 subtype-specific primers (B and D) were used. The RAZOR EX-analyses were performed under field conditions. A no template control (NTC) and a positive control were included in all runs (A and B). Real-time RT PCR amplification with the ABI 7300-instrument was done under standard laboratory conditions (C and D). Results obtained with the RAZOR EX-instrument under field conditions were in complete agreement with those obtained with the ABI 7300-instrument under standard laboratory conditions.

et al., 2009). A previous version of the RAZOR instrument has been used for the detection of *Vibrio cholera* (Koskela et al., 2009), *Bacillus anthracis*, *Brucella* spp., *Francisella tularensis*, and *Yersinia pestis* (Matero et al., 2011) and its applicability under near-patient field conditions has been clearly demonstrated.

Ct-values obtained by the ABI 7300 were consistently 3.5–6.5 cycles lower than those obtained by the RAZOR EX-instrument, similarly as has been described earlier (Koskela et al., 2009). Despite these differences in the algorithms to determine cut-off values, the actual methodological sensitivities, as determined by analysis of serial dilutions of template and the diagnostic accuracy, did not vary between the ABI and RAZOR EX-methods.

The results demonstrate the feasibility of a portable thermocycler designed for field use in the rapid diagnosis of influenza A-virus infections. This technology provides high sensitivity and specificity combined with speed and can be performed near the patient without the need for shipping of the sample to a diagnostic laboratory. This makes the RAZOR EX-method particularly suitable for outbreak investigations. In our hands the RAZOR EX-method exhibited equal assay sensitivity and specificity as compared to standard real-time PCR technology (ABI 7300). These assays could be simplified further by using pre-fabricated kits, e.g., lyophilized reagents in the reaction pouches, and development of assays for other important respiratory pathogens should be considered.

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Molecular Characterization of Adenoviruses Among Finnish Military Conscripts

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Although adenoviruses were identified as important respiratory pathogens many years ago, little information is available concerning the prevalence of different adenovirus serotypes, which are circulating and causing epidemics in Finnish military training centers. Over a period of five years from 2008 to 2012, 3577 respiratory specimens were collected from military conscripts presenting with symptoms compatible with acute respiratory tract infection. Upon initial testing for certain respiratory viruses by real-time PCR, 837 of these specimens were identified as adenovirus-positive. For 672 of these specimens, the serotype of the adenovirus responsible was successfully determined by DNA sequencing. Serotypes 1, 2, 3, and 4 were detected in 1, 3, 181, and 487 samples, respectively. Adenovirus epidemics were observed during each year of this study. Based on these findings, adenovirus vaccination should be considered for military conscripts in the Finnish Defence Forces. ***J. Med. Virol. 88: 571–577, 2016***.

KEY WORDS: respiratory infection; military training centers; real-time PCR; genetic sequence

INTRODUCTION

Upper respiratory tract infections are the most common infectious diseases among persons of all age groups and they are mostly caused by viruses. Occasionally these viruses reach the lower respiratory tract, causing bronchitis, bronchiolitis, and pneumonia, which can often be severe, even life-threatening. Respiratory viruses are easily transmitted in crowded conditions, such as in day-care centers, schools, and especially in military garrisons [Gray et al., 1999; World Health Organization, 2008; O'Shea and Wilson, 2013]. Overcrowding, physical and mental stress and frequent traveling may predispose an individual to infection with respiratory pathogens. Respiratory infections have been recognized for a long time as a common illness among military recruits during their service [Top, 1975; Sanchez et al., 2001]. Particularly in garrisons, epidemics caused by respiratory viruses are often characterized by a sudden onset, and the rapid identification of the pathogen may help in planning appropriate counter-measures and treatment strategies.

Adenoviruses are a common cause of acute respiratory diseases globally. For many years, adenovirus-associated acute respiratory illnesses have been reported among adults, including military populations [Gray et al., 2000]. Epidemics caused by adenoviruses can be associated with high levels of morbidity [Potter et al., 2012]. Adenoviruses can cause a wide range of clinical manifestations, ranging from mild to severe infections. The most common clinical presentations of adenovirus infections are pneumonia, bronchitis, upper respiratory tract infections, and common cold symptoms [Kunz and Ottolini, 2010]. Some adenoviruses are related to follicular conjunctivitis or pharyngoconjunctival fever and highly contagious keratoconjunctivitis [Lenaerts et al., 2008]. Among immunocompetent individuals, adenovirus infections are generally mild but sometimes, particularly in immunocompromised patients, adenovirus infections can cause severe disease [Tebruegge and Curtis, 2012].

Currently 60 human adenovirus serotypes are known. They are divided into seven subgroups (A to

Conflict of interest: None

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G) on the basis of hemagglutination features, DNA (deoxyribonucleic acid) homology, and genomic organization [Robinson et al., 2013]. The majority of adenovirus infections are diagnosed in children, but they are increasingly also recognized as illnesses in adults [Cesario, 2012]. Several serotypes have been associated with a variety of diseases in humans. Among adults, adenovirus subgroup B (serotypes 3, 7, and 21), subgroup C (serotypes 1, 2, and 5) and subgroup E (serotype 4) are common causes of respiratory tract infection and are frequently associated with outbreaks among civilians and in the United States (US) Armed Forces [Brosch et al., 2009; Lu et al., 2013].

Since the 1950s, it had been recognized that US military recruits are prone to adenovirus infections, particularly types 4 and 7. Many recruits infected with these adenoviruses develop pneumonia and require hospitalization [Sivan et al., 2007; Brosch et al., 2009; Hoke and Snyder, 2013]. A live oral vaccine to prevent infections with adenovirus types 4 and 7 was introduced in the early 1970s, and was shown to significantly reduce febrile respiratory illnesses in vaccinated recruits [Kolavic-Gray et al., 2002; Russell et al., 2006]. After years of successful use, vaccine production was discontinued by the only manufacturer in 1996, which resulted in a resurgence of adenovirus-induced respiratory illnesses among military recruits [Gray et al., 2000]. A large outbreak of adenovirus occurred at Fort Jackson in 1997, during which type 4 was isolated in 50 percent of 147 trainees hospitalized with acute respiratory disease. Deaths associated with adenovirus infections have adenovirus infections have [McNeill et al., 2000; Centers for Disease Control and Prevention (CDC), 2001; Kolavic-Gray et al., 2002]. Morbidity associated with adenovirus four rapidly increased during the years 1997–1998 as remaining vaccine stocks were progressively consumed. In 1999, when the vaccine was no longer available, adenovirus four quickly became responsible for 98 percent of all diagnosed cases of adenovirus infections in US military personnel [Russell et al., 2006]. Furthermore, the lack of adenovirus vaccine resulted in adenovirus outbreaks during 1997–2000 in US military training facilities, and high rates of adenovirus infection were reported [Kolavic-Gray et al., 2002]. With vaccine no longer available, efforts to control endemic spread and epidemics have been largely unsuccessful [Russell et al., 2006]. However, a new oral vaccine against adenovirus types 4 and 7 was approved by the US Food and Drug Administration in March 2011, and after a break of several years, the adenovirus vaccination program for military recruits resumed in October 2011 [Potter et al., 2012; Hoke and Snyder, 2013; Radin et al., 2014].

Respiratory viruses are the source of significant outbreaks of illness among military conscripts in Finland each year, commonly during late winter and early spring [Hulkko et al., 2010]. Finnish garrisons actively participate in the sentinel surveillance for viral respiratory infections coordinated by the Institute for National Health and Welfare (THL). This surveillance provides detailed information on the circulation of various respiratory viruses in the Finnish population as well as on the properties of these viruses. Outbreaks of influenza A and/or B are registered every year. Parainfluenza viruses 1, 2, and 3 as well as respiratory syncytial virus (RSV) usually cause very limited outbreaks or present as isolated cases [Hulkko et al., 2010]. However, the Finnish Defence Forces has had little information on the adenovirus serotypes circulating in Finland. The present study sheds light on the different adenovirus serotypes causing outbreaks in the Finnish Defence Forces over a period of 5 years. This information may serve in considerations as to whether or not adenovirus vaccine might reduce adenovirus-associated morbidity in military conscripts.

MATERIALS AND METHODS

Adenovirus Samples

Nasopharyngeal aspirates or combined flocked nasal and throat swab samples (Copan, Brescia, Italy) were collected by staff physicians or nurses, from military conscripts with acute respiratory infections (ARI) for sentinel surveillance of respiratory infections conducted by THL. Conscripts presenting with influenza-like illnesses (ILI) or with ARI were included in this surveillance. The WHO case definitions for ILI and ARI have been used to select patients from whom specimens have been collected. With strict adherence to the ILI definition, a number of adenovirus-positive patients would have been missed because some of them presented without or with only low fever. The samples were stored and transported refrigerated either by mail or by army courier services within one or two days of collection.

As shown in Figure 1, 24 military garrisons and border guard detachment units participate in this surveillance system. After initial screening for adenovirus, influenza virus types A and B, parainfluenzavirus types 1, 2, and 3, and for RSV by real-time PCR [Ronkko et al., 2011], the samples were stored at −70°C. For the present study, 794 adenovirus-positive samples collected during the years 2008 through 2012 were available.

Purification of Nucleic Acids and Real-Time PCR

Viral nucleic acids were extracted from 100 μl of original clinical samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Purified nucleic acids were eluted in a volume of 50 μl.

For primary diagnosis of adenovirus infections, respiratory samples were tested by a SYBR-Green PCR [Allard et al., 2001] from 2008 until November 2010. After that, a probe-based real-time PCR

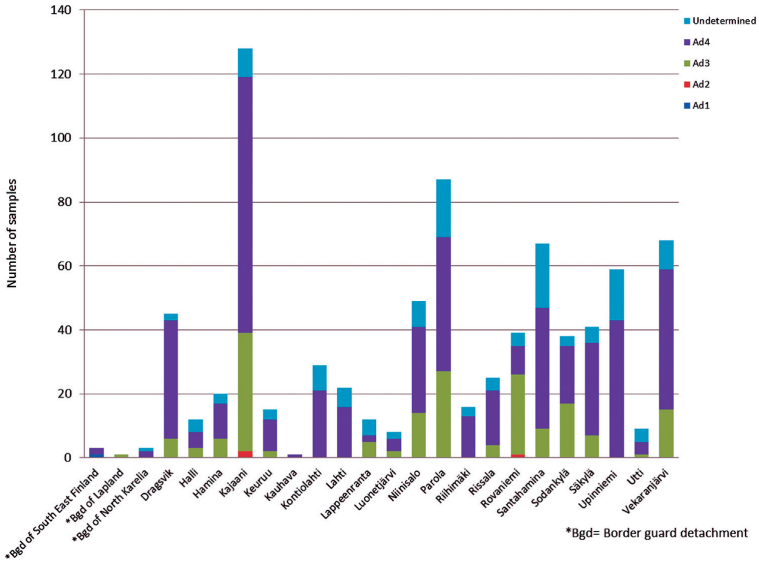

Fig. 1. Adenovirus-positive samples were collected from individuals with acute respiratory infections in 24 Finnish military garrisons or border guard detachments during the years 2008–2012. The serotype was determined for 672 specimens either by direct sequencing of a PCR amplicon or after cloning. For 122 samples the serotype could not be identified. The annual percentage of undetermined serotypes varied from 0 to 27.8 percent of all adenovirus positive samples during these years (Table II).

[Damen et al., 2008] was used. For identification of the serotype, all samples were tested by the SYBR-Green assay, amplifying a 301 base pair (bp 21–322) segment of the adenovirus hexon gene [Allard et al., 2001] (Table I). The thermal cycling profile consisted of 15 min at 95°C, followed by 45 or 55 cycles of 15 sec at 94°C, 30 sec at 55°C, and 30 sec at 72°C, and the Stratagene Mx3005P PCR platform using ABsolute Blue QPCR SYBR Green Low ROX Mix (ThermoScientific, Epsom, UK).

Cloning

Twelve samples from which direct sequencing of the amplicon gave inconclusive results were further analyzed by cloning the amplicon and subsequent sequencing. Amplicons were cloned using the TOPO TA cloning kit according to the manufacturer's instructions (Invitrogen Corporation, Carlsbad, CA). Six colonies from the cloning plate of each of the 12 samples were analyzed.

Sequencing and Genetic Comparison

Amplicons and cloning products were sequenced by the Finnish Institute for Molecular Medicine (Helsinki, Finland). Nucleotide sequences were further analyzed with the Sequencer 5.1 program (Gene Codes Corporation, Ann Arbor, MI). The BLAST search tool (http://www.ncbi.nml.nih.gov/BLAST/) and ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) were used to compare amplicon sequences to reference sequences published in GenBank.

RESULTS

A total of 3,577 respiratory samples were collected during 57 months over a total observation period of 60 months from 2008 to 2012. The vast majority of adenovirus-positive individuals were between 19 and 21 years of age, male, and in the military rank of conscript. The locations where the infections were diagnosed are shown in Figure 1. All samples were analyzed for the presence of influenza A and B, parainfluenza 1, 2, and 3, RSV and adenoviruses with real-time PCR [Ronkko et al., 2011]. Of those, 837 samples (23.4 percent) were found adenovirus-positive by real-time PCR (Fig. 2). During the period of observation, the majority of adenovirus-positive patients presented with febrile tonsillitis, but in contrast to patients with a streptococcal pharyngitis, palpable cervical lymph nodes were smaller. CRP and leukocytes usually were increased. Adenovirus-positive conscripts were excused from active service, typically for 2–3 days until they were afebrile. Major adenovirus epidemics were recorded during late winter and early spring. This is the period when conscripts

TABLE I. Primers of the SYBR Green Real-Time PCR -Assay for the Stratagene PCR Platform

Target gene	Primer sequences	Final concentration (nM) Stratagene	Amplicon length (bp)	Reference
hex1deg	5′-GCCSCARTGGKCWTACATGCACATC-3′	70	301	Allard, A et al 2001
hex2deg	5′-CAGCACSCCICGRATGTCAAA-3′			

are in basic training and participate in field exercises. Often these spring outbreaks of adenovirus are preceded by influenza outbreaks which often start within weeks of the new conscripts having entered service in January (data not shown). Because of the common influenza outbreaks, influenza vaccine has been offered to all conscripts entering service since 2012. In the two latter years of this surveillance, adenovirus epidemics also occurred in fall. These major outbreaks were caused either by adenovirus type 3 or type 4, with one of these types clearly dominating each outbreak (Table II). Adenovirus-positive samples were observed in 41 of the 57 months during which respiratory samples were obtained, with a monthly positivity rate for adenoviruses ranging from 0.6 to 69.4 percent. Of these 837 adenovirus-positive samples, 794 (94.9 percent) were available for identification of the serotype based on genetic analyses. Sequences obtained from the samples were aligned with those of reference viruses available from GenBank. The nucleotide sequences of the amplicon were heterogenous enough to allow discrimination of subgenera and serotypes [Allard et al., 2001]. Sequences from the study samples were 99–100 percent identical to those from the reference viruses of serotypes 1, 2, 3, or 4, respectively.

Serotypes 4 (487 samples) and 3 (181 samples) were most prevalent in this material. In addition to this, one virus of serotype 1, and three of serotype 2 were identified by direct sequencing of the PCR amplicon (Table II). Furthermore, the amplicon of 12 real-time PCR low-positive samples with a cyclic threshold value (Ct) $\geq$ 41 was cloned and the sequence was established from multiple colonies. All these 12 samples were from 2010 and early 2011 and were of serotype 4.

The serotype of 122 samples could not be determined by the methods used (Table II). All these undetermined samples had a negative or a weak positive result upon retesting by real-time PCR with Ct $\geq$ 42 in a PCR run of 55 cycles. 65 of these samples were PCR positive only with 55 cycles, and six of these undetermined samples remained real-time PCR negative upon retesting for this study.

The prevalence of the two most common serotypes 3 and 4 varied during the years of observation. Serotype 3 was dominant during 2010 whereas serotype 4 was the most common during the years 2008, 2009, 2011, and 2012 (Table II). Genetic difference between the four serotypes was found to be significant as, the amplified sequence varied from 4.2 to 22.3 percent as determined by alignment using Clustal W software.

DISCUSSION

Adenoviruses in army training centers are common health problems leading to considerable number of days off service. This study presents findings on the molecular characterization of adenoviruses circulating in Finnish garrisons where conscripts are trained. Using real-time PCR, sequencing, and cloning techniques, the presence of adenovirus serotypes 1, 2, 3, and 4 was verified in 794 samples collected from military conscripts during 2008–2012. Adenovirus serotypes 1, 3, and 4 were also detected in members of Border Guard Detachments over the same period of observation. In Border Guard Detachments housing conditions are less crowded than in garrisons.

The serotype of 122 samples (15.4 percent) of the 794 adenovirus PCR-positive samples could not be determined further with sequencing, possibly due to very low amount or partial degradation of viral DNA in the clinical sample. Most of the undetermined samples were, on initial testing, found to be PCR-positive with a probe-based real-time PCR, which yielded lower Ct-values than the SYBR Green assay i.e., reflecting higher assay sensitivity. In the present study, a SYBR Green-based PCR assay was used, as a probe assay may interfere in sequencing of the PCR product.

However, using cloning techniques, for 12 of these undetermined samples collected in late 2010 and early 2011 the serotype could be identified as type 4, even though serotype 3 was the dominant serotype during 2010.

Recently, Yliharsila et al., [2012] identified human adenovirus serotypes with an array-in-well-hybridization assay from the Finnish civilian population. In their study, a total of 231 adenovirus-positive samples were collected between April 2010 and April 2011. The age of patients included in the study ranged from 0 to 67 years, and adenovirus serotypes were detected in a variety of different specimen types (ocular, nasopharyngeal aspirate or swab, and other respiratory specimens (NP), stool or others). Their study showed that individuals aged 18–67 years were mostly affected by adenovirus serotypes 3 and 4. This is in agreement with the results presented here, as the specimens for the present study were obtained from conscripts with an average age of 19 years.

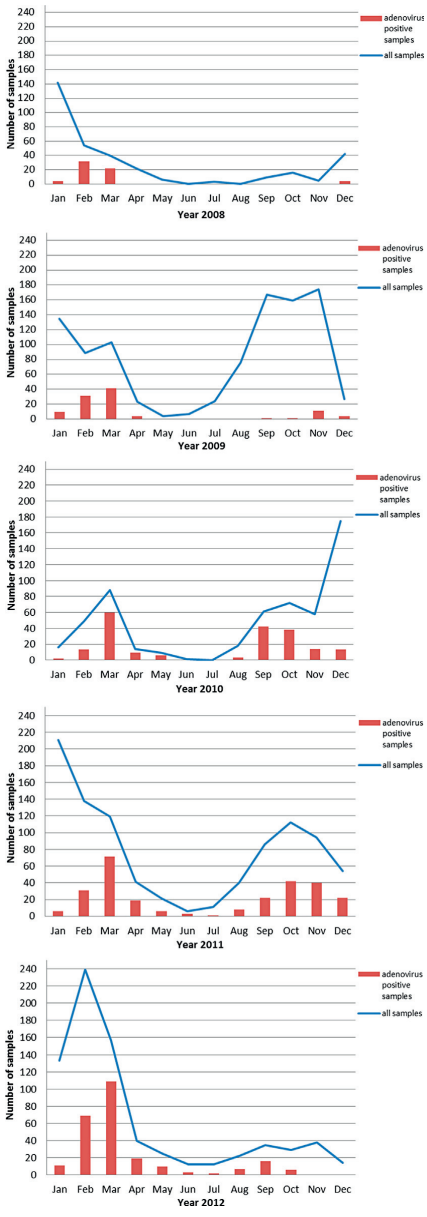

Fig. 2. From 3577 samples collected during the years 2008–2012, 837 samples (23.4 percent) were found to be adenovirus-positive upon initial testing by real-time PCR when the specimen was received by the laboratory.

Yliharsila et al., [2012] also found adenovirus serotypes 1, 2, 5, and 6, in addition to serotypes 3 and 4 in nasopharyngeal secretions taken from patients of all age groups. Other adenovirus serotypes (8, 19, 31, and 37) in their study were detected in clinical samples from patients with clinical presentations other than respiratory infection. They also observed an outbreak of adenovirus serotype 3 during 2010, which is in agreement with the results of the present study (Table II).

In addition to adenovirus serotype 4, also adenovirus serotype 3 presented as a major serotype in the sample material. Adenovirus serotype 3 is one of the most prevalent serotypes detected globally and has been described as causing both upper and lower respiratory tract infections, particularly in young adults [Lebeck et al., 2009; Kunz and Ottolini, 2010]. In the United States, serotype 3 has been reported as one of the key causes of acute respiratory disease among military conscripts [Top, 1975]. Adenovirus serotype 3 has also been linked to infections other than those of a respiratory nature, e.g., infections causing neurological symptoms [de Ory et al., 2013] and myocarditis [Treacy et al., 2010]. Adenovirus serotype 3 belongs to adenovirus group B, together with adenovirus serotype 7.

Adenovirus serotype 7 is included in the adenovirus vaccine used in the US Armed Forces, together with adenovirus serotype 4. The vaccine package includes two separate tablets, one containing adeno 4 active core and one adeno 7 active core [Hoke and Snyder, 2013]. However, serotype 7 was not identified from the material in the present study. Serotype 7 had been found in epidemics before the re-introduction of vaccination in October 2011 [Ryan et al., 2002]. Additionally, serotype 7 has also been identified as a common pathogen among conscripts in several locations [Jeon et al., 2007; Yusof et al., 2012; Yu et al., 2013]. Interestingly, serotype 7 started to present a significant threat in the US Armed Forces when the spread of serotype 4 was controlled by vaccination [Hoke and Snyder, 2013]. The new adenovirus variant of serotype 14 has been associated with several outbreaks of acute respiratory disease with high rates of morbidity both in the US and Europe [Gray and Chorazy, 2009; Tate et al., 2009; Carr et al., 2011; Hoke and Snyder, 2013], and inclusion of this serotype in the vaccine is under consideration. In 2007, an outbreak of serotype 14 occurred among military conscripts, and 48 percent of the patients with febrile respiratory infection were shown to be infected with this serotype. These infections resulted in many hospitalizations and one death [Tate et al., 2009]. Serotype 14 was not detected in Finnish conscripts during this study, or in the Finnish civilian population in the study carried out by Yliharsila et al., [2012].

An oral adenovirus vaccine was used for about 25 years before vaccine production was halted. After the adenovirus vaccination was discontinued,

TABLE II. Adenovirus Serotypes Detected in the 794 Samples Analyzed

		Typed by adenovirus real time PCR procedure				Typed by cloning	
Sampling year	Total specimens	Type 1	Type 2	Type 3	Type 4	Type 4	Undetermined serotype (%)
2008	55	0	0	11	44	0	0/55 (0.0)
2009	96	1	2	6	84	0	3/96 (3.1)
2010	178	0	1	133	33	8	3/178 (1.7)
2011	266	0	0	30	158	4	74/266 (27.8)
2012	199	0	0	1	156	0	42/199 (21.1)
Total	794	1	3	181	475	12	122/794 (15.4)

adenoviral illnesses among US military conscripts returned to high levels [Russell et al., 2006]. Blasiole et al., [2004] showed how the prevalence of certain adenovirus serotypes differed substantially during the years 1996 to 2002 in US military conscripts. Prior to the adenovirus vaccine discontinuation, adenovirus serotypes 4 and 7 made up only 4 percent each of verified cases, but when the vaccine was no longer available in 1999, adenovirus serotype 4 became responsible for 98 percent of adenovirus morbidity. Kolavic-Gray et al., [2002] presented a prospective epidemiological investigation of 678 military conscripts, where adenovirus serotypes 3, 4, and 21 were found to cause acute respiratory disease. Since the new adenovirus vaccine was taken into use at military basic training centers in October 2011, febrile respiratory illness and adenovirus serotype 4 rates have fallen dramatically [Hoke et al., 2012].

Until now, the Finnish Defence Forces have had little information on the prevalence of the adenoviruses circulating within Finnish garrisons. By genotyping adenoviruses, it was possible to identify which adenovirus serotypes occur during adenovirus epidemics in Finnish military garrisons. Since no adenovirus 7 infections were diagnosed during this 5-year follow-up, Finnish military conscripts might benefit from adenovirus virus vaccine containing the serotype 4, as is used in the US Armed Forces. This would, however, require continued surveillance to determine, whether adenovirus 7 or any other serotype would replace adenovirus 4 after wide-spread vaccination of conscripts.

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