Forensic Analysis of Human DNA from Samples Contaminated with Biological Weapons Agents

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The following work is dedicated to
my family and my partner.

Without your support, love and patience
this work would have never seen the light of day.

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RÉSUMÉ

Depuis plusieurs années, l'armement d'agents biologiques est considéré un problème pour les agences de sécurité. Ces dernières exigent des procédés alternatifs afin de manipuler des échantillons médico-légaux qui pourraient être contaminés par des armes biologiques.

Dans cette étude, deux méthodes d'extraction d'ADN, manuelle et automatisée, furent comparées pour leurs habilités de retirer des agents biologiques, mais aussi leurs efficacités et consistances lors de l'extraction d'échantillons contaminés avec des bactéries, spores et toxines. Les agents biologiques furent analysés à partir de l'ADN purifié afin d'en assurer une décontamination complète, ainsi que d'en vérifier leurs effets durant le processus d'isolation de l'ADN humain. Les résultats démontrèrent que l'incubation d'échantillons dans une solution de lyse cellulaire élimina les bactéries et les toxines. Par contre, une étape additionnelle de filtration de 0.22 µm fut nécessaire pour retirer les spores bactériens. Les échantillons de sang et de cellules épithéliales démontrèrent une réduction et/ou dégradation de l'ADN lorsqu'exposé à certaines bactéries. Le processus d'extraction automatisé serait préférable, par rapport au processus manuel, pour isoler l'ADN humain contaminé par des armes biologiques.

ABSTRACT

The use of biological agents as potential weapons has been a concern of security agencies for many years. Security agencies require alternative field protocols for handling forensic samples that could be contaminated with biological weapons.

In this study, manual and automated DNA extractions were compared for the ability to remove biological agents and for their effectiveness and consistency when samples were contaminated with bacteria, spores or toxins. Purified DNA was evaluated for the absence of the agents, and for the effects of the process on the isolated human DNA. Results demonstrated that incubation of samples in a cell lysis solution eliminated bacteria and toxins, but an additional 0.22 µm filtration step was necessary to successfully remove bacterial spores. Blood and buccal swab samples exposed to some bacteria showed DNA loss and/or degradation. The automated extraction procedure would be preferable over the manual protocol to isolate human DNA contaminated with biological weapons.

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GLOSSARY OF ABBREVIATIONS

ATCC:	American Type Culture Collection
ATR:	Anthrax toxin receptor
AVA:	Anthrax vaccine absorbed
BC:	Before Christ
BoNT/A:	Botulinum toxin A
BSA:	Bovine serum albumin
BWC:	Biological Weapons Convention
CB:	Columbia broth
CBRN:	Chemical, biological, radiological and nuclear
CDC:	Centers for Disease Control and Prevention
CE:	Capillary electrophoresis
CFU:	Colony forming unit
CL3:	Containment level 3
Cp:	Crossing point
cRNA:	Carrier RNA
DTT:	Deithiothreitol
EDTA:	Ethylenediaminetetraacetic acid
EF:	Edema factor (anthrax virulence factor)
ELISA:	Enzyme-linked immunoadsorbent assay
FAD:	Filtered, autoclaved and deionized (water)
GI:	Gastrointestinal
HPV:	Hydrogen peroxide vapour
LD_{50}:	Median lethal dose for a given population
LF:	Lethal factor (anthrax virulence factor)
INFγ:	Interferon-γ
MeOH:	Methanol
$MnSO_4$:	Manganese sulphate
MHC:	Major histocompatibility complex
NDDB:	National DNA Data Bank
NML:	National Microbiology Laboratory

OD:	Optical density
PA:	protective antigen
PBS:	Phosphate buffered saline
PBST:	Phosphate buffered saline with Tween-20
PCR:	Polymerase chain reaction
PHAC:	Public Health Agency of Canada
PTSAgs:	Pyrogenic toxin superantigens
PVDF:	Polyvinylidene fluoride
qPCR:	Quantitative real-time polymerase chain reaction
RCMP:	Royal Canadian Mounted Police
RFLP:	Restriction fragment length polymorphism
SEB:	Staphylococcal enterotoxin B
SDS-PAGE:	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOP:	Standard operating procedures
STDEV:	Standard deviations
STR:	Short tandem repeats
TCR:	T-cell receptor
TSA:	Tryptic soy agar
TSB:	Tryptic soy broth
TSST-1:	Toxic shock syndrome toxin-1
UK:	United Kingdom
US:	United States
VHF:	Viral hemorrhagic fever

CHAPTER I

INTRODUCTION

Biological warfare

In current times, the increasing threat of bioterrorism has lead security agencies to prepare more for encountering biological and chemical weapons at crime scenes. Pathogenic biological weapons have been used for centuries to disperse illness in humans, animals or plants and to propagate chaos in order to gain a strategic or tactical advantage over an adversary (Ghosh *et al.*, 2009; Kendall *et al.*, 2008). Over the years, a large number of lethal and non-lethal biological agents have been employed, including protozoa, fungi, bacteria, protists, viruses, and toxins derived from microorganisms and plants (Weinstein & Alibek, 2003). One of the most recent and well-known uses of a biological weapon is the scattering of *Bacillus anthracis* spores in a powder form through the United States (US) mail system. The twenty-two isolated cases of anthrax generated enormous expense and resulted in an high security alert for the country, and were successful in generating fear and panic in the population (Ghosh *et al.*, 2009; Kendall *et al.*, 2008; Weinstein & Alibek, 2003).

The application of biological weapons against a population and their interests, including crops, livestock, and wildlife, is not a novel concept. In fact, there are numerous references throughout history describing the use of biological agents. Ancient scriptures from the fifth century before Christ (BC) revealed that Scythian archers plunged their arrows in decomposing bodies or in blood mixed with manure to inflict contaminated wounds on their enemies (Gosh *et al.*, 2002; Kendall *et al.*, 2008). In the same century, there were also plentiful accounts of the use of plant toxins, venoms, and other poisonous substances applied to arrows or water supplies to create biological weapons (Kendall *et al.*, 2008; Mangold & Goldberg, 1999; Riedel, 2004). During the siege of Caffa by the Tartars in the Middle Ages, plague-infected cadavers with bubonic (*Yersinia pestis*) were flung over the castle walls using catapults. It has been speculated that this tactic may have been responsible for the advent of the Black Death in Europe (Gosh *et al.*, 2002; Kendall *et al.*, 2008; Mangold & Goldberg, 1999; Perry & Fetherston, 1997; Riedel, 2004, Wheelis, 2002). *Variola major*, also known as smallpox, has been used throughout history as a very effective biological weapon. During the French Indian War, the British distributed smallpox-contaminated blankets to immuno-naïve Native Americans loyal to the French, with the expectation of

causing an epidemic of smallpox and decimating their defences (Christopher & Ruoff, 1997; Gosh *et al.*, 2002; Riedel, 2004).

Prior to World War I, biological agents were not utilized in a systematic way, but with advances in the field of microbiology and the development of modern technologies, it became possible to manufacture significant quantities of various agents and potentially disperse them over large areas. For example, during World War I, Germany pursued a biological warfare program with *Bacillus anthracis* (anthrax), *Pseudomonas pseudomallei* (glanders), *Vibrio cholerae* (cholera) and *Yersinia pestis* (plague), but there is no evidence of its application (Gosh *et al.*, 2002; Kendall *et al.*, 2008; Riedel, 2004). Nevertheless, because of the large use of chemical weapons in this war, international diplomatic efforts (Geneva Protocol) prohibited the proliferation and use of weapons of mass destruction, which included biological warfare agents. Unfortunately, no verification process of compliance was addressed (Christopher & Ruoff, 1997; Gosh *et al.*, 2002; Kendall *et al.*, 2008; Riedel, 2004).

During World War II, Japanese forces operated a secret biological warfare research facility (unit 731) which carried out human experiments. They exposed prisoners of war to plague, anthrax, syphilis, and other agents as part of their program to develop and observe the diseases (Williams & Wallace, 1989; Riedel, 2004). In response to suspected biological weapons programs in other countries, the US, United Kingdom (UK) and Canada initiated their own biological warfare development programs which resulted in the weaponization of tularemia, anthrax, rinderpest, brucella and botulinum toxin. The mass production of these pathogens was instituted, and there were plans to incorporate these agents into bombs for distribution in enemy territories (Bryden, 1989; Miller *et al.*, 2001; Riedel, 2004).

In the years after the World Wars, the development of the biological warfare agents for offensive application was shifted to research for defensive purposes. From the development of vaccines to the vulnerability of cities to aerosolized agents, the researchers were preparing against imminent attacks (Gosh *et al.*, 2002; Kendall *et al.*, 2008; Riedel, 2004). Public safety issues were raised internationally because of unproven allegations reported in newspapers of propagation of biological agents by bioterrorists (Riedel, 2004).

As of 1972, many countries signed a treaty that prohibited the development, production, acquisition, transfer, stockpiling, and usage of any biological weapon. The rationale behind this treaty discussed during the Biological Weapons Convention (BWC), currently signed by 171 countries, was to supplement the 1925 Geneva Protocol (UNOG, 2010). However, many countries still pursue research into defence or protection against biological warfare agents, which are not prohibited by the BWC (Gosh *et al.*, 2002; Kendall *et al.*, 2008; Riedel, 2004).

Since the BWC, large scale production of biological weapons has been limited. However, numerous incidents against individuals or small groups involving biological agents have been recorded, largely associated with terrorist attacks (Gosh *et al.*, 2002; Kendall *et al.*, 2008; Riedel, 2004). Henceforth, the use of biological agents shifted from focus on war to bioterrorism. Historical bioterrorism events involving the use of biological warfare agents have been classified and divided into three categories: terrorist events, criminal events, and state-sponsored assassination (Tucker, 1999). An investigation of the Aum Shinrikyo, a Japanese apocalyptic cult, initiated after the death of twelve people in the Tokyo subway system by means of sarin gas was considered a terrorist attack. This lead to the discovery of previous unsuccessful biological attacks with aerosolized anthrax and botulinum toxin in Japan (Henderson *et al.*, 2002; Kendall *et al.*, 2008; Riedel, 2004; Tucker, 1999).

In 2001, five letters containing *Bacillus anthracis* spores were sent from Trenton, New Jersey, to government buildings and news media centers in the US. Twenty-two anthrax cases resulting from these attacks were confirmed or suspected, with eleven of these cases being authenticated as inhalational anthrax resulting in five deaths. Eleven cases were identified as cutaneous anthrax (Henderson *et al.*, 2002; Riedel, 2004; Weinstein & Alibek, 2003). It was reported that one letter addressed to Senator Daschle contained two grams of powder containing between 100 billion to one trillion spores per gram (Henderson *et al.*, 2002). The anthrax attack of 2001 has heightened concerns about the feasibility of large-scale aerosol biological weapons attacks by terrorist groups (Henderson *et al.*, 2002).

It is important that we understand the characteristics and logistics of biological weapons in order to shield the population from bioterrorism threats and actions. Agencies

need to anticipate the type of biological weapon that could be used, how and where it could be dispersed and find the best way to prevent panic and possible harm to the population. In a worst case scenario, the containment and decontamination of the site(s) would be required.

Characteristics of biological warfare agents

The ideal biological weapon has low visibility, high potency, accessibility, low production costs and can easily be delivered throughout a target population. Biological agents chosen by terrorist as weapons are typically found in nature, which make them cheap and easy to obtain. Potential agents are easy to grow and produce in large quantities without significant technological resources (Kendall *et al.*, 2008). The minor difficulty of developing a pathogen for a biological attack resides in attaining a way to spread the biological agent without degrading its infectivity or toxicity. Biological agents can be dispersed through air (i.e., aerosol generator), or through the water and food supply (Langford, 2004). Terrorists may also choose biological agents that are difficult to detect and where illness may not be apparent for several hours to several days after the dispersement (CDC, 2010).

In North America, biological agents have been divided into three risk categories depending on how easily they can be spread, the severity of the illness, or the death they cause (CDC, 2010). Category A represents the agents with the greatest threat to public and national security. These agents may be rapidly transmitted from person-to-person and require special public preparedness to prevent this spread. Panic and social disruption would be created due to the high death rates (CDC, 2010; Ghosh *et al.,* 2009; Rotz *et al.*, 2002; Weinstein & Alibek, 2003). Category B biological agents, labelled as a moderate threat to community residents, are the second highest priority and necessitate specific laboratory capacity and enhanced disease monitoring. The less severe illness and low death rates of these biological agents nonetheless promote fear (CDC, 2010; Ghosh *et al.,* 2009; Rotz *et al.*, 2002; Weinstein & Alibek, 2003). The last category, Category C, has the potential to become a major threat with the help of engineered modifications for mass distribution inside a targeted population. These emerging pathogens are easily available and produced, and spread and have the potential for high morbidity and mortality rates and major health impacts (CDC,

2010; Ghosh *et al.,* 2009; Rotz *et al.*, 2002; Weinstein & Alibek, 2003). Table 1 lists many infectious agents that have potential to be developed as biological agents.

TABLE 1 **Biological pathogens identified by US Centers for Disease Control and Prevention as likely to have been weaponized and likely to be used as biological terrorism threat agents (courtesy of Kendall *et al.*, 2008).**

Biological agents	Threat Category	Type of Microorganism	Natural Occurrence
Ebola virus	A	Virus	Anthroponosis*
Lassa fever virus	A	Virus	Anthroponosis
Marburg virus	A	Virus	Anthroponosis
Rift Valley fever virus	A	Virus	Zoonosis**– may be vector-borne
Variola major	A	Virus	Anthroponosis
West Nile virus	B	Virus	Zoonosis – primarily vector-borne
Yellow Fever virus	C	Virus	Zoonosis – primarily vector-borne
Bacillus anthracis	A	Bacterium	Zoonosis – may be vector-borne
Brucella	B	Bacterium	Zoonosis – may be vector-borne
Burkholderia mallei	B	Bacterium	Zoonosis
Coxiella burnetti	B	Bacterium	Zoonosis – may be vector-borne
Francisella tularensis	A	Bacterium	Zoonosis – may be vector-borne
Rickettsia prowazekii	B	Bacterium	Zoonosis – primarily vector-borne
Yersinia pestis	A	Bacterium	Zoonosis – primarily vector-borne

***An infectious disease that is spread from humans to animals.**
****An infectious disease that is spread from animals to people.**

Most of the highest risk agents are viruses. Viruses are a diverse class of infectious agents, varying in size and morphology, complexity, host range, and pathologic effect on their hosts. Because of the very wide range of diseases and pathologies attributed to viruses, and because there is little that can be done to interfere with the growth of viruses, many viruses are ideally suited for use as biological terrorism agents (Kendall *et al.*, 2008). However, one drawback to viruses is the additional technical manipulations required to grow and purify a suitable quantity to obtain the desired effect. Viruses, in contrast to bacterial pathogens, multiply only in living cells and are fully dependent on the abilities of the host cells (Murray *et al.*, 2009). Of the hundreds of viruses causing human diseases, viruses associated with hemorrhagic fevers are considered major threats. Ebola and Marburg are two of the eighteen different viruses that cause viral hemorrhagic fever (VHF) in humans and other primates (Kendall *et al.*, 2008). The Russian biological warfare program has

weaponized the Marburg virus, a Category A biological threat agent, and Iraq is also believed to have attempted weaponization of both Ebola and Marburg viruses. Any sporadic outbreak of these diseases on any continent other than Africa would be highly considered for a bioterrorist attack (Weinstein & Alibek, 2003). The smallpox virus is another Category A biological threat agent. Smallpox is a highly contagious and often fatal infectious disease (CDC, 2010; Rotz *et al.*, 2002). Smallpox was eradicated with worldwide vaccination, but the virus is still available for research and biological weapon development (CDC, 2010; Kendall *et al*., 2008; Weinstein & Alibek, 2003).

Many of the risk group A and risk group B agents are bacteria (Table 1). Bacteria are unicellular, prokaryotic microorganisms that possess cell walls. Their chromosomes are composed of DNA. They are capable of reproducing very rapidly, via cell division, and they have no reliance on other cells for growth. Some bacteria, for example *Bacillus anthracis*, are known to be able to store their DNA inside a spore as protection from environment dangers such as heat, chemicals and ultraviolet light (Christopher & Ruoff, 1997; Kendall *et al.*, 2008).

One example of a bacterium known to be used as a biological weapon is *Francisella tularensis* which produces the disease tularemia. Tularemia is a potentially serious illness that occurs naturally in North America, Europe, and Asia. This Category A biological threat agent is transmitted in a number of ways, including direct contact with bodily fluids from an infected animal host, through the feeding of arthropods, inhalation of aerosolized bacteria in water droplets, as well as through ingestion of contaminated food or water, but infection is not spreadable from person-to-person (Kendall *et al.*, 2008; CDC, 2010; Weinstein & Alibek, 2003). *Francisella tularensis* can survive for weeks at low temperatures in moist environments (Murray *et al.*, 2009). In addition to its environmental stability, the bacterium is considered a likely agent for bioterrorism because it can easily be aerosolized to enhance inhalation exposure. An infective dose may be as few as 10 to 50 bacteria (CDC, 2010; Kendall *et al.*, 2008; Weinstein & Alibek, 2003). Other bacteria in these risk group categories include *Bacillus anthracis*, *Brucella*, *Coxiella burnetti* and *Yersinia pestis.*

A third group of agents with potential for use as biological weapons are toxins. Biological toxins are poisonous substances, generally proteins, produced or derived from living organisms, such as bacteria, cyanobacteria, fungi, and some species of plants and aquatic organisms, that cause diseases or other debilitating responses in humans and other animals (Kendall *et al.*, 2008). Biological toxins that have been considered weapons or threat agents include staphylococcal enterotoxin B (SEB), botulinum toxin A (BoNT/A), ricin, and various mycotoxins (CDC, 2010; Langford, 2004; Kortepeter & Parker, 1999). Each toxin has a unique effect, but in general, toxins are capable of entering human cells and they generally disrupt protein production or functions. Toxins, being small particles, are easily dispersed in the environment by droplet aerosolization, distributed in water or food supplies or in concentrated pellets as described in the following examples:

In 1978, the "Umbrella Assassination" incident used a modified umbrella containing a ricin-filled pellet which was injected into the journalist Georgi Markov's thigh. He died in the hospital three days later. Another example of a toxin with the potential of being used as biological weapon is the paralytic poison saxitoxin. Saxitoxin, located commonly in shellfish, is produced by two marine dinoflagellates, *Gonyaulax catenella* and *Gonyaulax tamarensis*, but is also produced by a cyanobacteria, *Anabaena circinalis*. Its toxicity, ranging from 0.3 to 1.0 mg/person, is even more acute if aerosolized and inhaled. The median lethal dose (LD_{50}) of saxitoxin, the dose that will kill half of exposed individuals, is 2 µg/kg. The mechanism behind the paralytic symptoms is the blocking of the sodium channel of nerve cells, preventing normal cellular function. Supportive care is the only treatment available (Langford, 2004).

Forensic identification

In the past twenty years, bioterrorism preparedness and response offices were established in the Centers for Disease Control and Prevention (CDC) and at the Public Health Agency of Canada (PHAC) to increase the capacity to act against biological terrorism. Both agencies have been mandates to: "*provide overall public health preparation by planning, improving surveillance and epidemiologic capabilities, developing rapid*

laboratory diagnostics, enhancing communication, and medical therapeutics stockpiling" (Rotz *et al.,* 2002).

Since an escalation in bioterrorism has been noted in the last ten years, military personnel and scientific teams have been designated to be responsible for the protection of the population, while chemical, biological, radiological and nuclear (CBRN) response teams, developed by government agencies, have been put in place to manage and investigate these events (Kendall *et al.,* 2008; Wilkinson *et al.,* 2007). For this purpose, equipment and techniques were developed to rapidly gather evidence at a bioterrorism scene to process and reconstruct the events and to aid in identifying the perpetrators (Hoile *et al.,* 2010).

Typical crime scene evidence, such as latent fingerprints, documents, electronic data, DNA, hair and fibres, are collected during the investigation of a crime scene (Hoile *et al.,* 2010; RCMP, 2010; Wilkinson *et al.,* 2007). While fingerprints remain one of the most frequent and critical pieces of evidence, human DNA collected from biological samples, is an important tool in the identification of the culprits. Samples for the purposes of DNA isolation include blood, semen, saliva, and also trace DNA within latent fingerprints (Hoile *et al.,* 2010; RCMP, 2010; Wilkinson *et al.,* 2007). Generally, following the collection of biological evidence, the samples are sent to the forensic laboratory. However, for dealing with samples related to a bioterrorism attack, the forensic facilities may not be equipped to handle potentially contaminated samples (Hoile *et al.,* 2010; Wilkinson *et al.,* 2007). Nonetheless, the contaminated samples will eventually have to be removed from the crime scene, since the required equipment for DNA typing is not mobile. Therefore, to safely transfer these samples from the contaminated crime scene to the forensic laboratories, various decontamination methods must be used to render the area and the samples safe.

DNA analysis

DNA proof of identity was used by the Royal Canadian Mounted Police (RCMP) for the first time in Canadian history in 1989 for a suspect who was denying allegations of a sexual assault victim. As the years progressed, DNA warrant provisions were added to the Canadian Criminal Code for the purpose of forensic DNA analysis during criminal

investigations. Collected DNA evidence is processed at the RCMP and compared through the National DNA Data Bank (NDDB) to help the criminal investigator identify the responsible for violent crimes (RCMP, 2010). In order to obtain and interpret profiles of possible criminals, human samples must undergo various steps to isolate and process DNA in a forensic laboratory. These consist of (1) the isolation of DNA from samples collected at the crime scene, (2) quantification of the DNA, (3) amplification of specific regions on the human chromosomes, and analysis of the amplified DNA, and (4) comparison with possible suspects whose DNA has been stored in the NDDB or whose DNA has been collected as part of the investigation.

1) ISOLATION OF DNA. Concentrated pure human DNA can be obtained by using various extraction methods. The fundamental principles of DNA extraction consist of exposing DNA by breaking the cell outer membrane and nucleus, then separating DNA from proteins and lipids with alcohol plus detergent and optionally, proteases. Manual DNA extraction using standard organic solvents such as phenol-chloroform is the basic method (Kishore *et al.,* 2006; Reimann *et al.,* 2007). This time-consuming protocol is not suitable for high-throughput DNA laboratories. In addition, the process involves toxic materials, such as a buffered phenol-chloroform isoamyl alcohol solution, and multiple steps such as alcohol precipitation and centrifugation (Applied Biosystems, 2010; Kishore *et al.,* 2006; Sensabaugh & Blake, 1993). Another available method of extraction is the use of a chelating resin with high affinity for polyvalent metal ions. This resin binds metal ions on polar cellular components, such as DNA. The basic protocol is to denature DNA into single-stranded DNA by boiling the samples in a resin solution and then separating the resin bound to DNA from the rest of the mixture by centrifugation. Separation of DNA using resin has been improved through the use of magnets. Resin containing magnetic beads can isolate purified DNA by applying a magnetic field (Greenspoon & Ban, 2002). DNA is then washed and eventually eluted off the resin by heating in an elution buffer (Applied Biosystems, 2010; Bailey *et al.,* 2003). The resin can accommodate a pre-determined maximum quantity of DNA and becomes saturated when an excess of sample DNA is present. This technique, with some variable pre-treatment steps, can effectively isolate DNA from a variety of substances and sources even when samples are contaminated with materials, such as soil

(Bailey *et al.,* 2003; Komonski *et al.,* 2004; Sebastianelli *et al.,* 2008). The advantage of this procedure is that it prevents the degradation of DNA caused by high temperatures and low ionic solutions simply by covering the DNA molecules with chelating metal ions. The affinity of the chelating resin can be increased with the additions of ions, such as Ca^{2+}, Mn^{2+}, and Mg^{2+} (Applied Biosystems, 2010).

Because forensic laboratories are experiencing an increased demand to process DNA samples due to the successful application of DNA technology in examining evidence from a wider variety of crimes, significant case backlogs have been generated at various law enforcement agencies such as the RCMP (Montpetit *et al.,* 2005). To meet the legal demands of this type of identification, and the technical aspects of the process such as adequate DNA quantity, as well as obtain adequate purity to guarantee high-quality results in subsequent analyses, the RCMP uses the DNA IQ™ system (Promega), where all components required for the magnetic bead extraction have been incorporated into a liquid handling platform that allows for complete automation of the procedure (Bailey *et al.,* 2003; Frégeau *et al.,*2010; Crouse *et al.,* 2005; Komonski *et al.,* 2004; Reimann *et al.,* 2007). An automated DNA extraction liquid handling robotic workstation must be able to process all relevant forensic biological material and repeatedly produce extracted DNA that is comparable to DNA extracted using existing forensic standard protocols in terms of quality and quantity (Anslinger *et al.,* 2005).

Another automated system is the Advanced EZ1® BioRobot workstation (Qiagen), which is a compact, desktop instrument for the extraction of nucleic acids from one to six samples (Sebastianelli *et al.,* 2008; Barkham, 2006; Nagy *et al.,* 2005). The instrument uses similar silica-based technology as DNA IQ™, in conjunction with pre-sealed, disposable trays containing all the necessary reagents for extraction (Nagy *et al.,* 2005). Post-lysis, the extraction time is approximately twenty minutes. The Advanced EZ1® BioRobot also requires little manual input other than specifying the expected quantity of DNA extracted and the elution volume, since all forensic protocols are pre-programmed onto the operating card (Investigator Card). Previous RCMP coop work term experiments demonstrated that the Advanced EZ1® BioRobot was able to extract DNA from a variety of biological materials

(e.g., blood, saliva, trace, chewing gum and cigarette butts) on different substrates (e.g., swab, plastic, paper and glass). However, improvement was necessary to match the consistently high standards required in forensic laboratories for contaminated samples (Jason Timbers - unpublished results). In order to optimize the DNA yield during extraction and purification, carrier RNA (cRNA) was added in these experiments to low-blood dilution samples before extraction, as suggested by various authors (Kishore *et al.,* 2006; Sebastianelli *et al.,* 2008).

2) DNA QUANTIFICATION. After isolation, DNA must be quantified. There are many different ways to quantify DNA and each method has its advantages and disadvantages. A very simple method is measuring DNA by absorbance using a spectrophotometer at 260 nm. An optical density (OD) of 1.0 corresponds to a concentration of 40 µg/mL, but free RNA and nucleotides present in the solution could falsely increase the DNA concentration. DNA purity is determined by the ratio of values at 260 nm over 280 nm. Protein impurities are present if the 260/280 ratio is higher than 1.8 (Fattorini *et al.,* 2000). Another method previously used by forensic laboratories is slot blot quantification for single- and double-stranded DNA. The principle of this technique consists of probing DNA trapped on a nylon membrane with a human DNA specific probe. After several washes, chemiluminescence on the probes is detected in the DNA samples and compared to a set of standards. Slot blot quantification can detect as little as 10 to 20 pg of DNA (Butler, 2005).

The newest technology for specifically quantifying human DNA in a sample involves the use of quantitative real-time polymerase chain reaction (qPCR) where amplified DNA is detected by means of a fluorochrome. Measures of fluorescence during qPCR are proportional to the amount of starting DNA. Measurements are taken during every cycle of amplification of the targeted DNA. In order to quantify the DNA, the data obtained during the exponential phase (geometric) of amplification using fluorescent primers are compared to a standard curve. The human DNA concentration in the solution is calculated using the crossing point (Cp), which is determined when the amplification curve crosses a selected threshold value. Non-specific amplifications are evaluated by checking the melting temperature of each amplified product. The DNA quantification kit used by the RCMP is

based on this approach. This kit targets a 62 base intron on chromosome 5p15.33 and can quantify DNA in a range from 0.023 ng/µL to more than 50 ng/µL (Applied Biosystem, 2010; RCMP, 2010).

In order to proceed with the next steps in identification, the DNA must be of reasonable quality (low degree of degradation) and purity (minimized polymerase chain reaction (PCR) inhibitory factors), and of sufficient quantity (Alonso *et al.*, 2004; Applied Biosystems, 2010; Kline *et al.*, 2005). Low amounts of DNA could lead to gaps in DNA profiles (allele dropouts), which refer to the failure to detect an allele within a sample or failure to amplify an allele during PCR, which can result in the misinterpretation of DNA profiles. Too much DNA could produce a high fluorescence background, an increase in strand slippage or stutter effects (Alonso *et al.*, 2004; Kline *et al.*, 2005). These problems could result in the inability to identify culprits. The minimum amount of DNA required by the RCMP to proceed to amplification is 0.250 ng (Kline *et al.*, 2005; personal communication with Chantal Frégeau, RCMP).

3) DNA AMPLIFICATION AND ANALYSIS OF AMPLIFIED FRAGMENTS. After quantification, the DNA is subjected to amplification of chromosomal regions established as being appropriate for distinguishing one individual from another. The quantification results and the amplified DNA play an important role in the quality and specificity of the PCR amplification. Various components added to the amplification mix of the PCR have been reported to improve specificity and increase the fidelity of the amplification process. For instance, the addition of KCl, Mg^{2+} ions and decreasing the pH, increase the specificity and fidelity of amplification by protecting DNA strands against heat-induced cleavage. Also, amplification inhibitors can be removed with the addition of bovine serum albumin (BSA) (Applied Biosystems, 2010; Marguet & Forterre, 1998; Sensabaugh & Blake, 1993).

The process of human DNA identification consists of comparing DNA profiles generated from biological samples collected at a crime scene to a reference sample, generally blood or buccal sample from the suspect, or compared to genotypes in the NDDB. Although it was demonstrated during the human genome project that 99.9 % of human DNA sequences

are the same in every person, enough of the DNA is different to distinguish one individual from another. These different DNA regions are highly variable and are unlikely to show similar profile patterns between individuals during DNA analysis, except for monozygotic twins (Carroll & Ciaffa, 2007). Forensic identification focuses on these areas of variability.

There are many techniques developed over the years to perform DNA analysis, for example restriction fragment length polymorphism (RFLP), PCR analysis or short tandem repeats (STR) analysis. Mitochondrial DNA analysis or Y-chromosome analysis are used to examine the DNA that cannot be analyzed by other techniques (BERIS, 2009). RFLP analysis uses restriction endonuclease digestions, and Southern blot analysis. The technique is executed only with large quantities of biological material, and DNA degradation would greatly affect the analysis. Briefly, variable lengths of DNA fragments created during the enzymatic digestion of the samples are hybridized with selected DNA probes for identification. The fundamental principles for PCR analysis are the same as RFLP. However, smaller and degraded DNA samples are able to be amplified (BERIS, 2009).

STR analysis is used to evaluate specific loci within nuclear DNA where repeats of two to five bases exist (Applied Biosystems, 2010). Variability in the number of repeats within these loci can be used to distinguish one individual from another. Typically each STR allele will be shared by five to twenty percent of individuals, but by examining several STR regions, the analysis will be more discriminating (Applied Biosystems, 2010). The RCMP uses a standard of nine specific STR regions. The analysis of human STR loci is based on the lengths of PCR amplified products, which are determined by the number of repetitions at each locus. PCR products can be analyzed in a slab gel format or by capillary electrophoresis (CE). The AmpF*l*STR® Profiler Plus® PCR Amplification kit is a fluorescent PCR kit for human STR identification used by the RCMP. The kit contains the required components for PCR and nine pairs of primers specific to tetrameric STR regions for human identification, plus two gender determining region (amelogenin) (Table 2) (Applied Biosystems, 2010). The resulting PCR products are resolved by CE, and commercially available software automatically analyzes the collected data. The average probability of identification of an individual using 13 STR loci is 1 in 82 billion (Applied Biosystems, 2010).

TABLE 2 **Chromosomal location, allele size and dye label of each of the STR tetrameric locus and gender determining region used in the AmpF*l*STR® Profiler Plus® PCR Amplification kit manufactured by Applied Biosystems. (Courtesy of Applied Biosystems, 2010).**

Locus	Chromosome location	Size range (base pair)	Dye label
D3S1358	3p	114-142	5-FAM
vWa	12p12-pter	157-197	5-FAM
FGA	4q28	219-267	5-FAM
D8S1179	8	128-168	JOE
D21S11	21	189-243	JOE
D18S51	18q21.3	273-341	JOE
D5S818	5q21-31	135-171	NED
D13S317	13q22-31	206-234	NED
D7S820	7q11.21-22	258-294	NED
Amelogenin (X)	p22.1-22.3	107	JOE
Amelogenin (Y)	p11.2	113	JOE

Effects of biological contaminants on DNA isolation and analysis

Contamination is a major problem for the forensic community, particularly with DNA typing of human DNA. Dirt, fire debris and microorganisms are but a few of the contaminating factors found in crime scenes. In addition to the issues of contamination, biological evidence is usually scarce and contaminating factors may contribute to DNA degradation. Luckily, PCR-based methods have allowed the analysis of very small DNA quantities, but contamination could still be a problem during amplification of the DNA.

Some biological evidence samples yield DNA that is refractory to amplification on first pass. The inhibition can be due to the presence of polymerase inhibitors. Those enzyme-based interfering inhibitors can be found in pigments of blood stains (free heme), cigarette butts (tobacco tars) and clothing (indigo dyestuffs) (Sensabaugh & Blake, 1993).

Small degraded fragments of DNA or the loss of preferential larger alleles during the amplification of human DNA could result in the loss of important genetic information that could potentially be used in forensic investigation. The DNA repair process from hydrolysis, oxidation and nonenzymatic methylation is seen *in vivo*, but may play an important role in decaying biological samples at a crime scene by inducing mutations in the genetic material

(Fattorini *et al.*, 2000; Kobilinsky, 1992; Lindahl, 1993). However, post-mortem investigations have revealed that time plays a minor role in the degradation of DNA due to spontaneous base modification, strand scission and depurination (Fattorini *et al.,* 2000; Kobilinsky, 1992; Sensabaugh & Blake, 1993). Establishing degradation by integrating controls during DNA typing is important (Sensabaugh & Blake, 1993). Contact with a surface (fabric, flooring, soil, etc.), exposure to lighting, temperature, humidity and chemical or biological contaminants are contributing factors that limit the chemical stability of DNA (Kobilinsky, 1992; Sensabaugh & Blake, 1993).

Biological evidence may come into contact with various chemicals that effect human DNA. Organic solvents such as alcohol, gasoline and cleaning fluids seem to cause little or no damage to DNA, which is good, since ethanol plays an important role in washing steps during the extraction of DNA. Acids and alkalis, on the other hand, generate chemical modifications of DNA bases. Formaldehyde gas, detergents, bleach and hydrogen peroxide vapour (HPV) are used to decontaminate crime scenes contaminated with biological warfare agents, but they induce damage to the DNA. The damage is reflected in a concentration-dependent manner, except for hydrogen peroxide which has a stronger damaging effect at lower concentration (Kobilinsky, 1992; Sensabaugh & Blake, 1993).

When microorganisms are present during DNA extraction, the eluted mixture will contain human and microbial DNA. Indeed, primers in the kit are designed to amplify human DNA loci specifically and function even in the presence of microorganisms in the samples. The eluted human DNA should generate suitable yields of amplified DNA for analysis (Kupfer *et al.,* 1999). However, PCR inhibition or allele dropouts have been seen in DNA samples containing common vaginal microorganisms such as *Candida albicans* American type culture collection (ATCC), *Gardnerella vaginalis* (clinical isolate) and *Bacteroides fragilis* ATCC, which interfered with the reliability of PCR-based systems (Lienert & Fowler, 1992). Human STR kits tested for the ability to amplify regions from microbial DNA demonstrated no reactivity in human primers (Fernadez-Rodriguez *et al.,* 1996; Webb *et al.,* 1993). However, it was shown in one study that microbial DNA could be the source of an extra nonspecific amplification band observed when a specific locus typing (D1S80) was

carried out from forensic biological sample contaminated by six of the thirty microorganisms used, which were *Corynebacterium sp.* ATCC, *Streptococcus sanguis* ATCC, *Micrococcus luteus* ATCC, *Klebsiella pneumoniae* ATCC, and two strains of *Pseudomonas stutzeri* (Fernadez-Rodriguez *et al.,* 1996). It is known that microbial contamination with *Staphylococcus epidermis*, *Candida valida*, *Escherichia coli* and *Bacillus subtilis* can induce strand scission in human DNA as a result of microbial nucleases, but such samples may still provide acceptable DNA yields and profiles if human specific primers are used (Fattorini *et al.,* 2000; Kobilinsky, 1992; Sensabaugh & Blake, 1993). So, while there is some evidence that contamination with specific microorganisms can affect the isolation and analysis of human DNA for forensic purposes, the effects of many other microorganisms and potential biological weapons, such as toxins, on human DNA are unknown.

Crime scene potentially contaminated with biological weapons

In addition to the issue of DNA degradation due to the presence of contaminating microorganisms, investigators must also be concerned with the potential dangers of contaminating materials. There are no standard decontamination procedures established to date to deal with crime scenes where microbes and other biological weapons might exist. When a site is known to contain potentially deadly biological agents, decontamination of the entire area with selected chemicals seems to be the common choice to rapidly remove potential danger for the investigating teams and the population. The cost of such decontamination is substantial ($ 23 million to decontaminate the Hart Senate Office building in the US during the 2001 anthrax attacks), but is necessary to reinstate the services (Henderson *et al.,* 2002). Chemicals, such as formaldehyde gas, detergents, bleach and HPV are effective, but these reagents degrade human or bacterial DNA samples, as mentioned previously (Hoile *et al.,* 2010).

Another technique used to remove infectious threats from a crime scene would be to irradiate the entire area, though this method may miss well-concealed biological agents. However, moving materials from the contaminated area into a “safe zone”, such as a mobile laboratory, would guarantee direct contact to irradiation. Bacterial spores are the most resistant of biological agents, so the gamma dose for inactivating them is therefore of great

importance. However, the quantity of gamma rays required to eradicate the infectivity of spores may also reduce the quantity of DNA recovered (Hoile *et al.,* 2010). Currently, postal services in the US sterilize mail with electron-beam (E-beam) irradiation to prevent another anthrax attack, but the application of this type of irradiation on buccal cells has been shown to reduce the yield and quality of DNA by compromising longer DNA fragments, rendering the amplification of the whole-genome unfeasible, but a partial analysis possible (Castle *et al.,* 2003).

The approach of retrieving human biological samples from a crime scene and then decontaminating them prior or during analysis is relevant from a forensic perspective. There are various methods of decontaminating forensic evidence and multiple methods may be necessary for decontaminating different agents. Apart from the previously discussed chemical and irradiation methods, there is also physical decontamination. Physical methods consist of thermal decontamination, filtration or separation of contaminated samples. When samples are to be collected for isolation of human DNA, thermal decontamination involving heat-inactivation of the biological agent is not practical since boiling or autoclaving for long periods of time may destroy human DNA. Another option is filtration or separation, which is unable to inactivate the biological agents, but would retain larger agents, such as bacteria and spores, although viruses and toxins would pass through. Such a method has been proposed to decontaminate human DNA extracted from blood (see below), where a 0.22 µm filter step was included at the end of the purification process (Hause, 2007).

This work was carried out as part of collaboration between the PHAC laboratory and the RCMP laboratory, and the protocol was tested on human samples contaminated with *Francisella tularensis, Yersinia pestis* and spores of *Bacillus anthracis*. Results demonstrated that the use of high temperature, 56 ºC and 95 ºC, for thirty minutes during DNA extraction could not guarantee complete inactivation of contaminating vegetative bacteria or spores in various human biological samples. However, in 255 samples, filtration before or after DNA extraction was completed to remove the infectivity of the selected pathogens without damaging human DNA. Filtration of samples after DNA extraction was judged to be the superior method because there was less reduction in the amounts of isolated DNA.

Furthermore, this protocol would require less handling of samples prior to DNA extraction, which should reduce the possible cross-contamination with DNA from a technician during the manipulation of the contaminated samples (Hause, 2007). Many aspects of the proposed protocol were not analysed in detail. For instance, the effect of the lysis buffer and proteinase K alone on the selected pathogens, the ability to eliminate infectivity when higher numbers of microbes or bacterial spores were used, the effect of other types of biological weapons, such as toxins, on human DNA isolation, and the ability of the extraction process to inactivate these agents, the effects of prolonged exposure to biological weapons on human DNA, and the incorporation of an automated extraction process to the proposed methodology of DNA isolation at a contaminated crime scene. Therefore, new pathogens along with previously studied biological agents were chosen to extend the research of the effects of contamination with microbes and toxins on human DNA isolation.

It was clear at the beginning of this project that it would be impossible to analyse every biological agent a terrorist could possibly choose. Originally, the plan was to include three bacteria that fell into Category A. However, it became clear that acquiring all these agents would be extremely difficult. Hence, it was decided to use surrogate organisms and to include a new category of agents, i.e. toxins.

PSEUDOMONAS AERUGINOSA & STAPHYLOCOCCUS AUREUS. *Pseudomonas aeruginosa*, a Gram-negative aerobic rod, is a bacterium that causes disease in animals and humans alike. Its natural habitat is in soil and water, but it may also be found on skin and artificial apparatuses. This bacterium is known for its ability to survive and grow in the environment with minimal nutrients. *Staphylococcus aureus* is a Gram-positive, facultative anaerobic coccus that is a commensal of humans. This bacterium is associated with local skin lesions, but can cause systemic disease. *Staphylococcus aureus* produces many toxins, one of which is a major cause of food poisoning (Prescott *et al.,* 2003).

YERSINIA PSEUDOTUBERCULOSIS. There are fifteen species in the genus *Yersinia*, which are Gram-negative and non-spore-forming coccobacilli, but only three forms are pathogenic to mammals, including humans. They are *Yersinia pestis, Yersinia*

pseudotuberculosis and *Yersinia enterocolitica* (Zhou & Yang, 2009). Of these, *Yersinia pestis* is the bacteria most likely to be developed as a biological weapon (Kendall *et al.*, 2008; Perry & Fetherston, 1997). *Yersinia pestis* can be easily retrieved from nature, as it is present in small rodents and soil. The World Health Organization reports 1,000 to 3,000 cases of plague, mostly bubonic form, worldwide every year (CDC, 2010). There are three types of plague: bubonic, septicemic and pneumonic. The bubonic plague symptoms are generally characterized by the presence of swelling in the lymph nodes of the armpits, neck, or groins. These swellings are known as buboes. Additionally, bubonic plague is associated with headaches, abdominal pain, bloody diarrhea and vomiting. Bubonic plague symptoms can rapidly progress to septicemic plague symptoms, which are fever, rapid heart rate, severe headache, vomiting, delirium and death. Symptoms of pneumonic plague are fever, weakness, and rapidly developing pneumonia with shortness of breath, chest pain, cough, and sometimes bloody or watery sputum (CDC, 2010; Henderson *et al.*, 2002). Without early treatment, pneumonic plague usually leads to respiratory failure, shock, and rapid death. There is a 70 to 100 % mortality rate for pneumonic plague (Zhou & Yang, 2009). To reduce the risk of death, antibiotics must be given within 24 hours of the first symptoms (CDC, 2010).

Depending on environmental conditions during the scattering of this biological weapon, the pathogen could survive for up to one hour in the atmosphere, even though it is easily destroyed by sunlight and drying, and decades in the soil (CDC, 2010; Zhou & Yang, 2009). After one to six days of an aerosol attack, pneumonic plague would be observed (CDC, 2010; Henderson *et al.*, 2002; Weinstein & Alibek, 2003). This acute infection can be transmitted from person-to-person, and so can spread widely because the symptoms are delayed (CDC, 2010; Henderson *et al.*, 2002; Weinstein & Alibek, 2003).

Due to difficulties acquiring *Yersinia pestis*, it was decided that *Yersinia pseudotuberculosis* could serve as a surrogate. Based on sequence analysis, the divergence of *Yersinia enterocolitica* from *Yersinia pestis* and *Yersinia pseudotuberculosis* occurred between 41 to 186 million years ago, whereas *Yersinia pestis* and *Yersinia pseudotuberculosis* are thought to have diverged about 1,500 to 20,000 years ago (Achtman

et al., 1999; Zhou & Yang, 2009). In fact, *Yersinia pestis* is a clone that evolved from *Yersinia pseudotuberculosis* shortly before the first known pandemic of human plague (Achtman *et al.*, 1999). *Yersinia pestis* has developed specialized strategies for virulence in hosts and transmission by flea-vectors, and many of these determinants are harboured in the genome of *Yersinia pseudotuberculosis* (Atcham *et al.*, 1999). Thirteen per cent of the genes in *Yersinia pseudotuberculosis* tend to be inhibited in *Yersinia pestis* (Atchman *et al.*, 1999). Only two chromosomal regions seem to be specific to *Yersinia pestis*, the regions which code for the virulence and transmission by fleas (Zhou & Yang, 2009). Through lateral acquisition, *Yersinia pestis* has also obtained two unique virulence plasmids, pPCP1 and pMT1. The pPCP1 plasmid is essential for bubonic and primary pneumonic plague, because it encodes a plasminogen activator that promotes the dissemination of peripheral infection (Zhou & Yang, 2009). pMT1 is essential for blocking phagocytosis by means of a capsule-like antigen, called F1 antigen, activated only at 37 ºC; the optimal growth temperature of *Yersinia pestis* is 26 ºC. pMT1 also encodes a murine toxin which facilitates the survival of the bacteria in the flea gut by promoting the production and activity of the phospholipase D (Zhou & Yang, 2009).

As described above, the evolution of *Yersinia pestis* from *Yersinia pseudotuberculosis* changed the symptoms and vectors of the disease, but the majority of the mechanisms were left unaltered. The mild disease caused by *Yersinia pseudotuberculosis* is transmitted through a food-borne route. Typical symptoms include fever and right-side abdominal pain. In rare cases, the disease may cause skin conditions, joint stiffness and pain, or spread of bacteraemia (Zhou & Yang, 2009). Antibiotic treatment is used to cure the infection. The LD_{50} of *Yersinia pseudotuberculosis* injected intravenously in mice is 10 Colony Forming Units (CFU) and this is comparable to the infectious dose of *Yersinia pestis* in mice (Zhou & Yang, 2009).

BACILLUS ANTHRACIS. *Bacillus anthracis* is a large encapsulated Gram-positive bacteria with aerobic capacity and no motility. This pathogenic bacterium is responsible for the disease known as anthrax, also named as the charcoal disease and black bane (Fouet & Mock, 2006; Ghosh *et al.*, 2009, Henderson *et al.*, 2002; Kendall *et al.*, 2008; Plomp &

Malkin, 2009; Weinstein & Alibek, 2003). It is thusly named because of the painless black ulcers with a swelled rim that appear on the skin. Approximately 20,000 cases of endemic anthrax in animals and humans occur annually in many regions of the world, such as Asia, Africa, South America and Australia (Henderson *et al.*, 2002). Under unfavorable growth conditions, such as high temperature or depletion of nutrients, *Bacillus anthracis* has the ability to produce spores (CDC, 2010). Once the conditions are favourable for growth and reproduction, the spore can develop into an active form (i.e., vegetative bacteria) (Ghosh *et al.*, 2009; Kendall *et al.*, 2008). *Bacillus anthracis* spores can survive for decades in the environment and are resistant to heat, microwaves, and ultraviolet light (Henderson *et al.*, 2002; Weinstein & Alibek, 2003). In addition, anthrax spores can survive days to weeks in the air (Weinstein & Alibek, 2003). The spore is covered by a loose-fitting layer called the exosporium (Plomp & Malkin, 2009), which gives the spore a size of approximately 1 µm (Henderson *et al.*, 2002). During the germination and growth of anthrax spore within a low iron medium, for example in soil, the spores secrete siderophores (Wilson *et al.*, 2010). Siderophores are ferric ion specific chelating agents that scavenge iron from the environment to provide nutriments to the pathogen (Neilands, 1995). The siderophores produced are petrobactins, which are required during early infection, and bacillibactins, which are required during the late stages of infection of *Bacillus anthracis* (Wilson *et al.*, 2010). The spores or its vegetative form grow well on ordinary laboratory media at 37 ºC, and germinate in an environment rich in amino acids, nucleosides and glucose, such as a host cell (Henderson *et al.*, 2002). Vegetative bacteria have poor survival outside of an animal or human host (Henderson *et al.*, 2002).

Bacillus anthracis spores can readily be dispersed in a population. The disease can be initiated with only a few spores, but 8,000 to 10,000 spores are required to make exposure fatal (CDC, 2010). As depicted in Figure 1, after the exposure of a host to *Bacillus anthracis* spores, macrophages will phagocytose the spores, where they will germinate and proliferate into the vegetative form of the bacterium (Kendall *et al.*, 2008; Henderson *et al.*, 2002; Weinstein & Alibek, 2008). The bacteria will then escape the macrophage by cytolysis and spread the toxic components inside the cell. The virulence factors of *Bacillus anthracis* consist of an antiphagocytic poly-γ-D-glutamate capsule and two toxins, which are

responsible for toxemia and septicemia (Fouet & Mock, 2006; Ghosh *et al.*, 2009 Weinstein & Alibek, 2008). The two toxins are delivered inside the eukaryotic cells where binding of the protective antigen (PA) takes place on the anthrax toxin receptor (ATR) in the cell wall (Fouet & Mock, 2006; Henderson *et al.*, 2002). A complex created by a protease will enable the delivery of the lethal factor (LF), a zinc protease, and the edema factor (EF), an adenylate cyclase, into the cell (Fouet & Mock, 2006; Ghosh *et al.*, 2009; Kendall *et al.*, 2008; Henderson *et al.*, 2002). LF is the most dangerous protein of the two, because one of its main functions is to block the immune response of the body (Kendall *et al.*, 2008). Anthrax disease caused by the active or spore form of *Bacillus anthracis* is divided into three categories: cutaneous anthrax, gastrointestinal (GI) anthrax and inhalational anthrax (CDC, 2010; Ghosh *et al.*, 2009, Henderson *et al.*, 2002; Kendall *et al.*, 2008; Weinstein & Alibek, 2003). Epidemics of zoonotic anthrax disease are occasionally seen in herbivores and can be transmitted to humans through the handling of livestock or the consumption of animal products, but it is not transmissible from person-to-person (CDC, 2010; Ghosh *et al.*, 2009; Henderson *et al.*, 2002; Kendall *et al.*, 2008). The first symptom appearing within days of exposure to cutaneous anthrax is a small sore that develops into a blister. The blister then develops into a painless skin ulcer with a black area in the center (CDC, 2010; Kendall *et al.*, 2008). If not treated, cutaneous anthrax can lead to death, with a mortality rate of up to 20 % (CDC, 2010; Ghosh *et al.*, 2009; Henderson *et al.*, 2002). Eating contaminated flesh can lead to GI anthrax. Symptoms appear three to five days after consumption and vary from nausea, loss of appetite, bloody diarrhoea, and fever followed by bad stomach pain. If not properly treated, death may occur with a fatality rate of 25 to 60 % (Ghosh *et al.*, 2009; Henderson *et al.*, 2002; Weinstein & Alibek, 2003). The infamous reputation of anthrax comes from the high mortality rate of the inhalational form of the disease, which can be as high as 80 % (Ghosh *et al.*, 2009; Henderson *et al.*, 2002). It appears from reported cases that symptoms may not be evident for up to two months after exposure (CDC, 2010; Kendall *et al.*, 2008; Henderson *et al.*, 2002). Inhalational anthrax symptoms resemble a flu-like illness with sore throat, fever, tiredness, shortness of breath, and muscle aches (CDC, 2010). It is important to note that the mortality rates in humans vary with different strains of *Bacillus anthracis* (Kendall *et al.*, 2008).

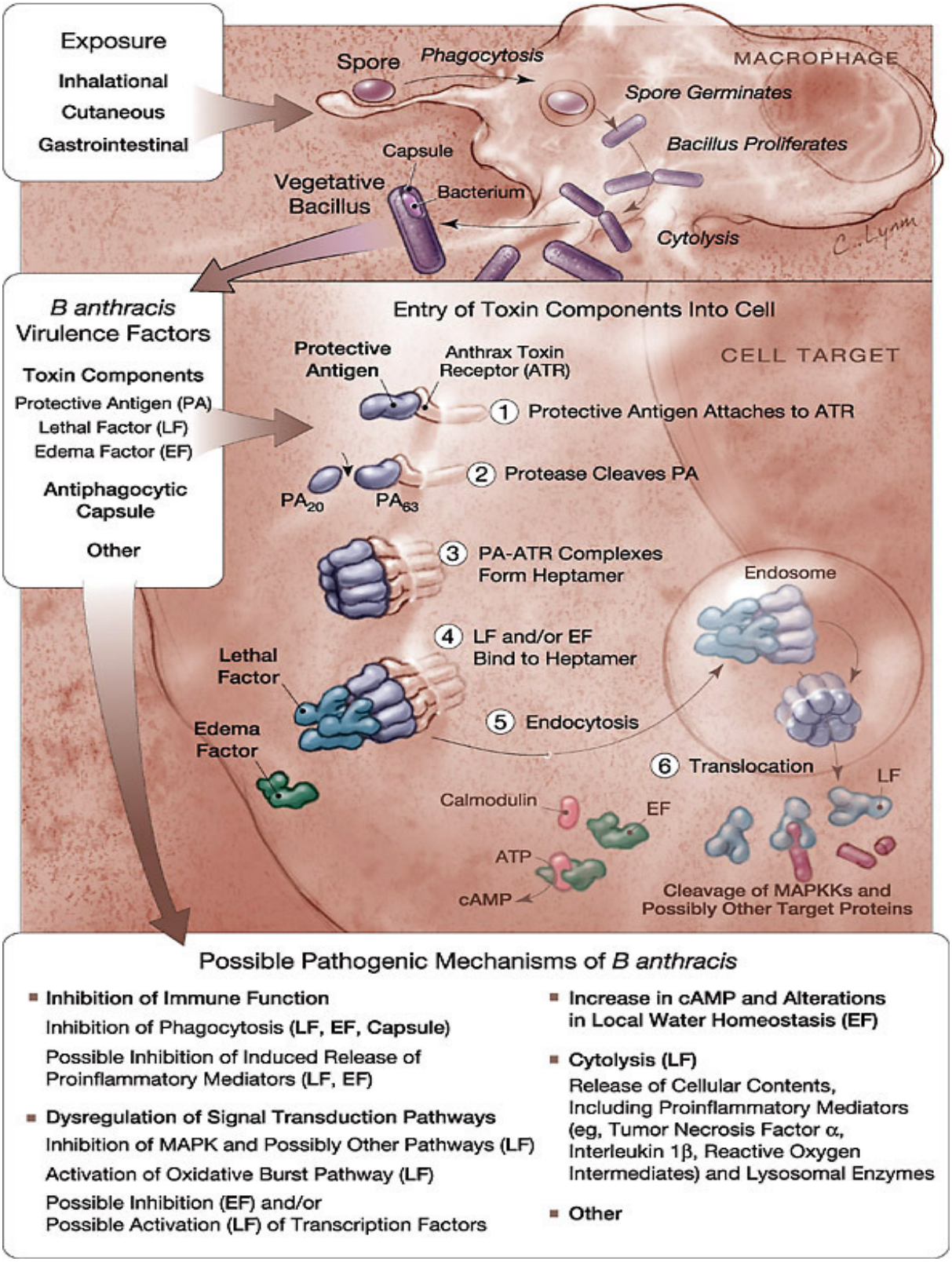

FIGURE 1: **Virulence factors of a bacterial spore of *Bacillus anthracis* after being inhaled, ingested, or touched by a mammalian host (courtesy of Henderson *et al.*, 2002).**

A recombinant protective antigen vaccine, known as the anthrax vaccine absorbed (AVA), is available for prevention of the disease to designated personnel, such as veterinarians, that are significantly at high risk of anthrax exposure. The Sterne strain of *Bacillus anthracis* is attenuated and has also been used to create a vaccine. Otherwise, prompt treatment using antibiotics over a period of 60 days is administered to prevent the late germination of inhaled spores. However, success of the treatment is dependent upon the exposure dosage and the route of infection (CDC, 2010; Ghosh *et al.*, 2009; Henderson *et al.*, 2002; Kendall *et al.*, 2008).

BOTULINUM TOXIN A. Historically, BoNT/A was the first microbial toxic substance to be licensed for medical use. A diluted version of the toxin is used to treat cervical torticollis, chronic neck pain and various dystonias, migraine, stroke and traumatic brain injury (Ghosh *et al*., 2009; Henderson *et al.,* 2002; Shukla & Sharma, 2005). However, BoNT/A is also known as a category A biological weapon that causes a muscle-paralyzing disease, botulism (CDC, 2010; Kendall *et al.,* 2008). Botulinum toxin A is a major threat to society, because it is easy to produce, transport, disperse, and demands prolonged intensive care of the victims (Ghosh *et al.,* 2009; Henderson *et al.,* 2002). As little as one gram could kill 1 million people if dispersed via aerosolization, which makes BoNT/A the most poisonous substance known to man (Carter *et al.,* 2009; CDC, 2010; Ghosh *et al.,* 2009; Kendall *et al.,* 2008; Sebaihia *et al.,* 2007; Shukla & Sharma, 2005).

Clostridium bacteria are Gram-positive anaerobic bacilli which some are able to produce these series of toxins, and have the ability to form spores tolerant to boiling (Ghosh *et al.,* 2009; Henderson *et al.,* 2002; Weinstein & Alibek, 2003). These soil-dwelling bacteria are easy to isolate from their natural habitat (Ghosh *et al.,* 2009; Henderson *et al.,* 2002). In an oxygen-deprived environment, such as preserved canned food, *Clostridium botulinum* spores can germinate and produce toxins (Ghosh *et al.,* 2009; Henderson *et al.,* 2002; Weinstein & Alibek, 2003).

The disease caused by *Clostridium botulinum* is the result of the action of botulinum toxins. There are seven antigenic forms of the botulinum toxin that are classified as type A to

G based on lack of cross-neutralization, but only four of them can cause human disease: toxin A, 54 % of disease; toxin E, 27 %; toxin B, 15 %; & toxin F, 2 % (Cater *et al.,* 2009; DasGupta, 2006; Ghosh *et al.,* 2009; Kendall *et al.,* 2008; Henderson *et al.,* 2002; Shukla & Sharma, 2002; Sebaihia *et al.,* 2007). The others (C, D, & G) have been reported in rare cases in humans, but usually affect other mammals and some fishes (Ghosh *et al.,* 2009; Kendall *et al.,* 2008; Shukla & Sharma, 2005). Bacteria within the *Clostridium botulinum* species are classified into groups based on the ability of the organism to digest complex proteins. Group I *Clostridium botulinum* strains produce botulinum toxins A, B or F; Group II strains generate type B, E or F toxins; Group III strains produce type C or D, whilst Group IV strains will produce only type G toxin (Sebaihia *et al.,* 2007).

The botulinum toxins are produced as two polypeptide chains; a heavy chain (100 kDa) linked to the light chain (50 kDa) by a single disulfide bridge (DasGupta, 2006; Henderson *et al.,* 2002; Shukla & Sharma, 2005). The heavy chain is subdivided into a binding domain, which irreversibly binds to cell surface receptors and a translocation domain, which mediates the transport of the light chain domain across the cell membrane (Shukla & Sharma, 2005). The light chain is a zinc endopeptidase that contains a catalytic domain that cleaves a specific protein (SNARE) responsible for the transport of the neurotransmitter, acetylcholine, within cells prior to its release. Lack of acetylcholine results in sagging muscle paralysis (DasGupta, 2006; Ghosh *et al.,* 2009; Kendall *et al.,* 2008; Henderson *et al.,* 2002; Shukla & Sharma, 2005; Weinstein & Alibek, 2003). The enzymatic proteolytic characteristics of *Clostridium botulinum* bacterial toxins, also produced by unique strains of *Clostridium baratii* and *Clostridium butyricu,* are responsible for their potency (Henderson *et al.,* 2002). The effects of botulinum toxin observed in the neuromuscular junction can be seen in Figure 2.

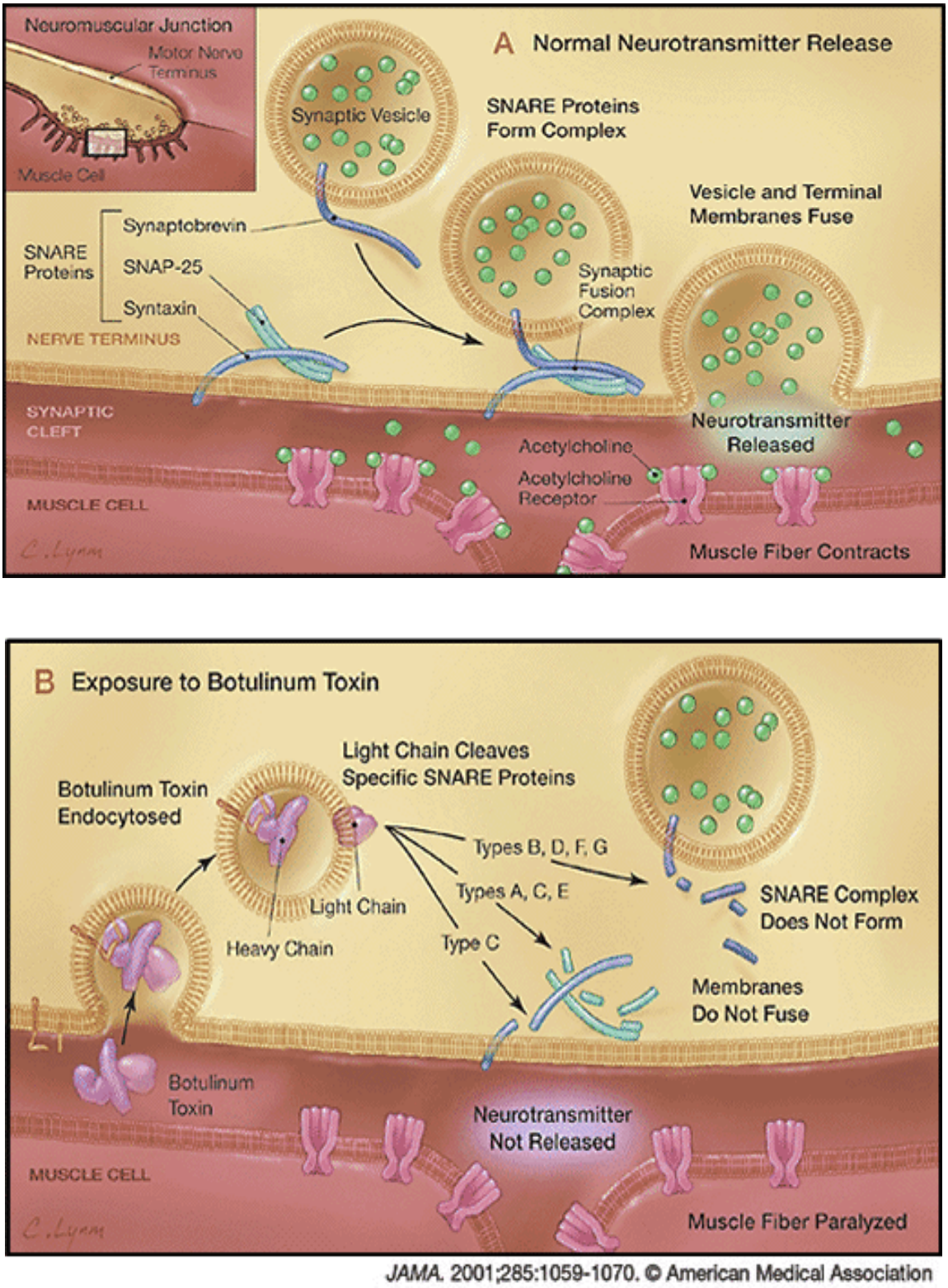

FIGURE 2: **Normal neurotransmission of acetylcholine inside the neuromuscular junction (A) and the mechanism of action of botulinum toxin A when exposed to the motor nerve terminus (B) (Courtesy of Henderson *et al.,* 2002).**

There are 140 cases of botulism annually in the US (Kendall *et al.,* 2008), and these occur from three different routes of exposure, cutaneous wounds, GI and inhalation. Skin contact with the toxin would not cause botulism (Ghosh *et al.,* 2009; Henderson *et al.,* 2002), and wound botulism caused by BoNT/A, and in some reported cases by BoTN/B, occurs only when the pathogen enters a deep wound (CDC, 2010; Shukla & Sharma, 2005). Food-borne botulism in infant occurs from consumption of contaminated water or food stocks, and in these cases, the pathogen will colonize the GI track and secrete the neurotoxins BoNT/A and BoNT/B (CDC, 2010; Kendall *et al.,* 2008; Shukla & Sharma, 2005; Weinstein & Alibek, 2003). Inhalational botulism, which is most likely to be selected by bioterrorist, is caused by the spreading of the bacterial spores or aerosolization of purified toxins into the respiratory system (Ghosh *et al.,* 2009; Kendall *et al.,* 2008; Henderson *et al.,* 2002; Shukal & Sharma, 2005; Weinstein & Alibek, 2003).

Once BoNT/A is absorbed via a wound or the mucosa, the neurotoxin spreads via the bloodstream, and will result in neurological symptoms, such as blurred vision and speech, and paralysis of skeletal muscle with normal mental function (CDC, 2010; Ghosh *et al.,* 2009; Henderson *et al.,* 2002; Weinstein & Alibek, 2003). Symptoms will manifest themselves within 12 to 72 hours after exposure to the toxin, and, if not treated, progressive weakness and paralysis of the respiratory organs could lead to death (CDC, 2010; Ghosh *et al.,* 2009; Henderson *et al.,* 2002; Kendall *et al.,* 2008; Weinstein & Alibek, 2003)

A botulism outbreak can be treated with antitoxin if detected rapidly. The passive immunization by these neutralizing antibodies requires timely administration in order to decrease further damage, however once the toxin is bound to the target cells in the body, the treatment is ineffective (Ghosh *et al.,* 2009; Kendall *et al.,* 2008; Henderson *et al.,* 2002; Shukal & Sharma, 2005; Weinstein & Alibek, 2003). Supportive care, such as gastric lavage and activated charcoal may aid the recovery of patients with food-borne botulism (Weinstein & Alibek, 2003). When water or food stocks are thought to be contaminated, they should be heated at 85 ºC for at least five minutes to inactivate the toxins (Ghosh *et al.,* 2009; Henderson *et al.,* 2002).

As mentioned previously, BoNT/A, by weight, is the most toxic substance known to man, with a LD_{50} of only 0.001 µg/kg when aerosolized, which means as little as 30 to 100 ng of the neurotoxin can be fatal (Carter *et al.,* 2009; Kendall *et al.,* 2008; Henderson *et al.,* 2002; Weinstein & Alibek, 2003). However, the toxin is light sensitive and is not resistant to extreme temperature and humidity, which can lead to the degradation of the toxin (Henderson *et al.,* 2002).

STAPHYLOCOCCAL ENTEROTOXIN B. *Staphylococcus aureus* is the second most common bacterial cause of food poisoning. This bacterium is a facultative anaerobic, Gram-positive coagulase-positive coccus that produces and secretes a broad range of exotoxins that play a part in colonization and disease in mammalian hosts (Dinges *et al.,* 2000; Roberts *et al.,* 2000; Weinstein & Alibek, 2003). Most of these proteins are enzymes and cytotoxins, such as hemolysins, nucleases, proteases, lipases, hyaluronidase and collagenase, and their purpose is to acquire nutrients for bacterial growth by lysing local host cells (Dinges *et al.,* 2000). Within this group, staphylococcal enterotoxins (SEA, SEB, SED, etc.) and toxic shock syndrome toxin-1 (TSST-1), act as pyrogenic toxin superantigens (PTSAgs) (Dinges *et al.,* 2000; Kendall *et al.,* 2008; Roberts *et al.,* 2000). Of these, SEB has been classified as a category B biological weapon, and so was chosen for this study (CDC, 2010; Kendall *et al.,* 2008).

SEB is encoded within a pathogenicity island that is regulated via three mechanisms: through a gene regulator (*arg*), a staphylococcal accessory gene regulator (*sar*), and the catabolite repression system (Dinges *et al.,* 2000). After transcription and translation, the exoprotein is cleaved into a small, mature, unglycosylated polypeptide molecule with a molecular weight of 28 kDa (Dinges *et al.,* 2000; Kendall *et al.,* 2008; Roberts *et al.,* 2000). The ellipsoid shape of SEB is generated by the linking of two domains, A and B, via a disulfide bridge. This structure, which can withstand boiling, renders the protein stable (Dinges *et al.,* 2000; Roberts *et al.,* 2000). The shape also enables the polypeptide to cross epithelial cell membranes and interact with the intestinal intraepithelial lymphocytes. Thereafter, SEB will bind to the Vβ chains of the T-cell receptor (TCR) on CD4 or CD8 T-cells and the major histocompatibility complex (MHC) class II molecule, resulting in the

production of activated T-cells, interferon-γ (IFNγ) and cytotoxic activity (Roberts *et al.,* 2000).

After one to six hours, immunostimulation against the consumed toxin of *Staphylococcus aureus* will induce food poisoning symptoms such as acute self-limited gastroenteritis, vomiting, abdominal pain, and diarrhea (Roberts *et al.,* 2000; Kendall *et al.,* 2008; Weinstein & Alibek, 2003). Inhalation of SEB, which is a more likely way of using it as a biological weapon, can result in minor respiratory symptoms that can last for one or two weeks (Kendall *et al.,* 2008; Weinstein & Alibek, 2003). The infective dose of the aerosolized SEB is 0.03 µg/kg, while the LD_{50} when consumed is approximately 1.7 mg/kg (Dinges *et al.,* 2000; Ghosh *et al.,* 2009; Kendall *et al.,* 2008; Roberts *et al.,* 2008; Rusnak *et al.,* 2004.). Supportive care is the only available treatment. Presumably, SEB illnesses could generate septic shock and death if consumed/inhaled in high doses (Kendall *et al.,* 2008; Weinstein & Alibek, 2003).

RICIN. Castor plants *(Ricinus communis*) produce castor beans, which contain a protein, ricin that is highly toxic when consumed (Barnes *et al.*, 2009; CDC, 2010; Kendall *et al.*, 2008; Shyu *et al.*, 2002; Weinstein & Alibek, 2003). Approximately 91 million metric tons of castor beans are collected annually to produce castor oil. Ricin, a toxalbumin, is available in large quantities worldwide due to the production of castor oil. The straightforward process to isolate the ricin protein broadens the potential for the use of ricin as a biological weapon (Barnes *et al.,* 2009; Kendall *et al.,* 2008). This category B cytotoxin can be readily distributed to any population by means of inhalation, injection or ingestion (Kendall *et al.,* 2008; Ghosh *et al.,* 2009; Weinstein & Alibek, 2003). Upon entering the body, the incubation period is six to eight hours before symptoms appear (CDC, 2010; Weinstein & Alibek, 2003). Ingestion of ricin result in vomiting and bloody diarrhea, and within several days organ failure and death (CDC, 201; Kendall *et al.,* 2008). Symptoms of inhalation are respiratory distress, due to pulmonary edema, heavy sweating and low blood pressure, which could eventually lead to the collapsing of the circulatory system, shock and death (CDC, 2010; Kendall *et al.,* 2008; Weinstein & Alibek, 2003). Death from ricin poisoning could take place within 36 to 72 hours post-exposure (CDC, 2010; Ghosh *et al.,* 2009; Weinstein &

Alibek, 2003). The LD_{50} of aerosolized or injected ricin toxin ranges from 3 to 5 µg/kg (Barnes *et al.,* 2009; Ghosh *et al.,* 2009; Shyu *et al.,* 2002).

The observed symptoms of ricin poisoning are caused by the toxin's capacity to inhibit protein synthesis through inactivation of the ribosome (Barnes *et al.,* 2009; CDC, 2010; Leshin *et al.,* 2010 Weinstein & Alibek, 2003). Fifteen hundred ribosomes can be inactivated by a single ricin molecule (Kendall *et al.,* 2008). Ricin is produced as two subunits of equal size, the A & B chains, which are linked by a disulfide bond. (Barnes *et al.,* 2009, Kendall *et al.,* 2008, Shyu *et al.,* 2002). The bridge between the two subunits is not essential for the A chain activity, but is necessary for entry into the cell, and therefore for toxicity (Barnes *et al.,* 2009;). The B chain, which has lectin properties, has the ability to bind to galactosyl residues on the cell surface, and is responsible for cell entry of the toxin via endocytosis (Barnes *et al.,* 2009; Leshin *et al.,* 2010; Shyu *et al.,* 2002). Prevention of toxicity can be achieved by inactivating the toxin at 70 ºC, the temperature at which the ricin protein irreversibly unfolds (Barnes *et al.,* 2009; CDC, 2010). If exposed to ricin, there is no treatment, but if the patient survives after five days on supportive care, the chances for survival increase (CDC, 2010; Kendall *et al.,* 2008; Weinstein & Alibek, 2003).

Ricin toxoid can be generated by heating, treating the toxin with chemicals or genetic recombination. The treatments render the proteins less dangerous to humans by weakening or suppressing the toxicity, while other immunologic properties are maintained. Such proteins are used in the development of vaccines. As the acquisition of ricin for use in experiments completed as part of the thesis proved difficult, and due to the high toxicity of this protein, a recombinant ricin toxoid that had been inactivated by the addition of extra amino acids in the linker chain was substituted.

Purpose

The purpose of this research project was to further characterize and improve a preliminary protocol for removal of bacterial agents and toxins from samples prior to DNA analysis, which would generate DNA of adequate quantity and quality to meet RCMP standards for human identification based on DNA.

Hypotheses

The main hypothesis of the project was that a standard protocol for DNA extraction would denature toxins and bacteria. Consequently the protocol previously developed for elimination of infectious bacteria from biological samples during extraction of human DNA, is adequate for removal of infectious material, including bacterial spores, without compromising human DNA samples.

A second hypothesis was that the use of a small robotic instrument and automated protocols, in a field situation, would provide higher and more consistent DNA yields compared to manual protocol.

Objectives

The project can be divided into four objectives:

1. To confirm that a preliminary protocol developed for the removal of infectious bacteria from human biological samples by using a 0.22 µm filter after DNA extraction can eliminate or inactivate all infectious material using higher concentrations of bacteria than previously tested.

2. To determine if a standard DNA extraction protocol denatures toxins.

3. To compare the quantity and quality of DNA extracted using two silica-coated magnetic bead extraction methods: an automated robotic system and a manual extraction using an existing RCMP protocol.

4. To determine if prolonged exposure of biological samples to live bacteria or toxins will affect DNA yields.

CHAPTER II

MATERIALS & METHODS

Bacteria

Frozen stock of *Pseudomonas aeruginosa* (ATCC 15442) and *Staphylococcus aureus* (ATCC 6538) were donated by Dr. Syed Sattar from the University of Ottawa [Department of Biochemistry, Microbiology and Immunology, *Ottawa, Ontario, Canada*]. A freeze-dried culture of *Yersinia pseudotuberculosis* was purchased from the ATTC (ATCC 11960) [*Manassas, Virginia, US*]. The avirulent *Bacillus anthracis* Sterne strain (34F2) was kindly provided, in the spore format only, by Mr. Denis Laframboise from the PHAC [*Ottawa, Ontario, Canada*]. The virulent form of *Bacillus anthracis* (bovine) (NML 03-0191) was obtained from the National Microbiology Laboratory (NML) [*Winnipeg, Manitoba, Canada*].

To prepare stocks of *Pseudomonas* and *Staphylococcus* bacteria, 1 mL of thawed bacteria was added to 100 mL of tryptic soy broth (TSB) which was prepared following the manufacturer's recipe (30 g/L) [*BD Difco™; Sparks, Maryland, US*]. Cultures were incubated on a shaker overnight at 37 ºC. Stocks were aliquoted with glycerol 50 % (1 mL) and stored at -80 ºC. A fresh overnight culture was inoculated from a frozen stock for subsequent experiments. For *Yersinia pseudotuberculosis*, the entire pellet was rehydrated with 1 mL of TSB. The aliquot was transferred into 5 mL of TSB, thoroughly mixed, and several drops were inoculated onto tryptic soy agar (TSA) plates, which were prepared following the manufacturer's recipe (40 g/L) [*BD Difco™*]. The agar plates were incubated at 37 ºC for 48 hours and then colonies were inoculated into broth, incubated, aliquoted and stored in the same fashion as described above.

A rapid assay to determine the concentration of vegetative bacteria was to compare bacterial absorbance at a wavelength of 600 nm with the log of CFU. For each hour, within a period of 24 hours, the growth of the bacteria was tracked by serially diluting the bacteria in TSB. At each point in time, including time zero, the OD at a wavelength of 600 nm was measured using a spectrophotometer. The blank used was TSB. After measurements, 100 µL of the aliquots (x3/bacteria) were plated on TSA and left for an overnight incubation at 37 ºC. Colonies were then observed and counted. Bacteria per millilitre were then determined following the universal formula:

$$\frac{Numbers\ of\ CFU}{Volume\ plated\ x\ Total\ dilution\ used}$$

The OD values equivalent to numbers of CFU could then be determined from a graphic of log CFU versus OD. These graphs were used in subsequent experiments to determine the quantity of bacteria added to the sample.

To generate stocks of spores from *Bacillus anthracis*, a protocol from the NML was followed. Briefly, *Bacillus anthracis* (NML 03-0083) was inoculated into a 250 mL flask containing 1:10 Columbia broth (CB). Media was prepared from powdered media [*BD Difco*™] at 35 g/L in sterile water where manganese sulphate ($MnSO_4$) was added to a final concentration of 0.1 mM. The flask was incubated at 37 ºC for 72 hours on an orbital shaker at 150 to 200 cycles/minute. The bacterial cells were pelletted by centrifugation (20 minutes, 4150 xg, 4 ºC) and washed three times with 50 mL of sterile water. After the last centrifugation, the pellet was resuspended in 10 mL of sterile water. This suspension contained both vegetative bacteria and spores. Vegetative bacteria were heat-inactivated by incubation at 80 ºC for ten minutes. The remaining solution of spores was plated on TSA and incubated overnight to determine the spore counts (1 colony = 1 spore) and then the stocks were adjusted to a concentration of 10^9 CFU/mL. Even though, many spores or vegetative bacteria could be counted in one colony. Finally, aliquots were stored at 4 ºC. For experiments conducted at PHAC, stocks of spores had been prepared by PHAC personnel and were adjusted to the appropriate quantity (10^6 and 10^9 spores/sample) prior to experiments.

All work with infectious *Bacillus anthracis* was carried out in containment level 3 (CL3) laboratories at the PHAC or at the University of Ottawa. At the end of the experiments, representative aliquots of 100 µL of all DNA samples extracted from contaminated biological samples and half the volume of extracted samples (50 µL) were incubated for one week on TSA to ensure no residual infectivity prior to removal from the CL3 laboratory for further analysis. To exit the CL3 laboratory, DNA sample tubes were wrapped in paper towel previously soaked in bleach (2 % sodium hypochlorite) for

ten minutes. DNA sample tubes were then placed in a sealed box as specified by CL3 standard operating procedures (SOP).

Toxins

BoNT/A (Sigma B8776) and SEB (Sigma S4881) were purchased from Sigma-Aldrich Canada Ltd. [*Oakville, Ontario, Canada*]. Both toxins were hydrated with sterile water to yield stocks of 1 mg/mL. The toxin solutions were stored at 4 ºC based on manufacturer's recommendations.

Rather than work with ricin, a toxoid was kindly provided by Cangene [*Winnipeg, Manitoba, Canada*]. This toxoid (TST 10088) is a modified form of ricin which has lost its toxicity due to the addition of an extra covalent linker between the A and B chains. Therefore, this modification prevents the toxoid from properly functioning, but does not prevent it from being recognized by antibodies to ricin. The stock received from Cangene (0.86 mg/mL) was diluted to a concentration of 300 µg/mL and stored at 4 ºC.

SDS-PAGE

For electrophoresis of proteins, 12.5 % sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used, and electrophoresis was carried out as per LaemmLi (LaemmLi, 1970). Gels were run at 100 V for one or two hours in a standard running buffer (pH 8.8).

Samples were mixed with 2X LaemmLi sample buffer (0.125 M of Tris, pH 6.8, 4 % SDS, 10 % β-mercaptoethanol, 20 % glycerol, and 0.004 % bromophenol blue) and boiled for a minute, prior to loading. Gels were fixed (5:4:1, H_2O:Methanol (MeOH):acetic acid) and stained with Coomassie blue (one tablet of R250 was diluted in 1 L of fixative solution) [Fisher, *Ottawa, Ontario, Canada*]. After mixing, the Coomassie Blue solution was filtered to remove excess particles. Gels were destained, as necessary, in a mixture of water, glacial acetic acid and MeOH (7:1:2). Molecular weight markers were included in every gel (PageRuler™ Prestained Protein Ladder Plus SM1811) [Fermentas Canada Inc., *Burlington, Ontario, Canada*].

ELISA

Enzyme-linked immunoadsorbent assays (ELISA) were performed with commercial kits specific for the detection of BoNT/A and ricin (BioThreat Alert® ELISA kits) [Tetracore® *Rockville, Maryland, US*]. Kits came with wells pre-coated with antibodies for the respective toxin, plus control wells without capture antibodies. Briefly, plates were blocked with blocking buffer overnight at 37 ºC (5 % skim milk powder in phosphate buffered saline (PBS) with 0.1 % Tween-20 (PBST) and washed four times with PBST prior to adding varying dilutions of samples to be tested. A standard curve of the toxin alone and toxin mixed with blood was included in all assays, as were negative controls of blocking buffer alone. Aliquots (100 µL) of toxin solution or negative controls were added to each well. Plates were incubated at 37 ºC for one hour in the dark and then washed four times with PBST. The conjugated antibody was added as per the manufacturer's directions. Plates were then incubated for another hour at 37 ºC. Finally, the peroxidase substrate (100 µL) was freshly mixed and added to each well. After a final incubation at 37 ºC for thirty minutes, plates were read by an ELISA microplate reader [BioTek EL311 SL, *Winooski, Vermont, US*] at a wavelength of 405 nm.

Sample preparation

Before each experiment, working surfaces were decontaminated with 70 % ethanol. Samples were manipulated with sterile forceps which were decontaminated using 80 % ethanol before and after each use, to prevent cross-contamination. While working with DNA samples, a surgical mask and latex gloves were worn to prevent any contamination of the samples with self DNA.

Blood from two anonymous donors at the RCMP was generously provided in a BD vacutainer® vial with anti-coagulate (5.4 mg of K_2 Ethylenediaminetetraacetic acid (EDTA)) for each set of experiments. Blood was refrigerated at 4 ºC and used for a maximum period of one week. Prior to its use, the blood was shaken for fifteen minutes. Blood dilutions were prepared in PBS to generate aliquots of 10 µL containing the equivalent of 0.1 to 10 µL of neat blood. Blood was applied to Puritan metical sterile cotton-tipped applicator and left to air-dry in a close sterile Petri dish for one hour. Epithelial cells from the inside cheeks of a

donor were collected via a buccal swab, which were air-dried for an hour in the same fashion as blood. Bacteria, spores or toxins were added in various amounts to the blood and buccal swabs. The volume of bacteria and spores never exceeded 100 µL (10^9 pathogen/samples), whilst volume of toxins never exceeded 10 µL. Following the application, the contaminated swabs were left to dry and then incubated at room temperature for varying times, ranging from one day to one week, prior to isolation of human DNA.

Control samples for each set of experiments (12 controls per biological agent) were prepared simultaneously to the blood and buccal samples. For extraction negative controls and confirm that bacterial DNA would not produce detectable results, three swabs containing the relevant biological agent without any trace of human DNA were prepared. Positive controls were whole blood samples (10 µL) on swabs without any added biological agents, also prepared in triplicate. For PCR negative controls and ensure no contamination of the PCR reagents and consumables, three empty sterile sample tubes and three blank swabs dipped in PBS were included. Once samples and controls were ready for DNA extraction, they were placed in sterile 2 mL screw-cap tubes (EZ1 DNA Investigation kit, [Qiagen, *Mississauga, Ontario, Canada*]) and refrigerated at 4 ºC prior to extraction, except for the time-exposure experimentation samples, which were incubated at room temperature for various periods of time up to one week.

Cell lysis

DNA was extracted from samples using commercially available kits with some modifications, including the use of the RCMP lysis buffer (100 mN NaCl, 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.5 % sarkosyl and 40 mM dithiothreitol (DTT)) for cell lysis. Prepared swabs were incubated overnight at 56 ºC in 341.3 mL of the lysis buffer to which Proteinase K [Qiagen] was added to make a final concentration of 1.2 mg/mL. Swabs were then placed in a spin basket (Spin-eZe™ baskets) [Fitzco Inc., *Spring Park, Minnesota, US*] and centrifuged at 9,300 xg for five minutes in order to collect liquid. The spin baskets and swabs were then discarded and cRNA [Qiagen] was added to all samples at a final concentration of 1 µg/µL. In some experiments, samples were split into two fractions of approximately 150 µL prior to extraction to reduce the number of samples to process.

DNA extraction

Manual extractions were carried out using the DNA IQ™ kit purchased from Promega [*Madison, Wisconsin, US*]. DNA IQ™ resin (12 µL/aliquots) was added to each of the samples. The tubes were briefly vortexed to mix the resin with the DNA and left at room temperature for at least five minutes. DNA IQ™ Lysis Buffer (700 µL/sample) [Promega] was then added, and samples were incubated at room temperature for fifteen minutes while being mixed by vortexing, every minute. After incubation, the sample tubes were pulse-spun for twenty seconds. The samples were then placed on a magnetic separator stand to attract the resin. The resin was washed twice with DNA IQ™ Magnetic Bead Wash (200 µL/sample) [Promega] and samples were spun, vortexed and returned to the magnetic separator stand for ninety seconds. The washes were carefully discarded without disturbing the resin. The tubes were left for ten minutes at room temperature with the lids open to allow for residual wash solution, containing alcool, to evaporate prior to the addition of DNA IQ™ Elution Buffer (100 µL) [Promega]. The samples were vortexed, spun and incubated at 65 ºC for eight minutes twice. The isolated DNA in the elution buffer was transferred to a clean 1.5 mL sterile tube.

Automated DNA extractions were carried out using the robotic workstation EZ1® Advanced [Qiagen]. Cartridges containing all necessary reagents (i.e. lysis buffer, elution buffer, resin, etc.), filtered tips and tip holders were provided in the EZ1® DNA Investigation Kits [Qiagen]. These were placed into the EZ1® Advanced workstation along with the sample tubes. Then, the DNA purification program automatically proceeded for an approximate time of twenty minutes, resulting in a final volume of 100 µL of DNA from each sample in Tris-EDTA buffer. After the removal of the eluates, the instrument was cleaned with 70 % ethanol and decontaminated with a twenty minute ultraviolet light sweep.

In experiments where manual and automated extracted samples were filtered, as a final step, the samples were passed through a low-binding Durapore® polyvinylidene fluoride (PVDF) 0.22 µm membrane by centrifugation as specified by the manufacturer (Ultrafree®-MC centrifugal filter) [Millipore, *Billerica, Massachussetts, US*]. Half of each of the filtered sample (50 µL) during DNA quantity and quality experiments was tested for the

presence of contamination with bacteria, spores or toxins. The remainder of the sample was stored at -20 ºC until DNA typing analysis. During the first testing of the protocol, the entire volume (100 µL) was tested during for each step of the extraction protocol.

DNA quantification

DNA quantification, PCR amplification, and CE were carried out at the RCMP Forensic Laboratory in Ottawa. DNA was quantified using the *Quantifiler® Human DNA Quantification* kit (Applied Biosystems, [*Foster City, California*, *US*]). This kit detects only human DNA in samples using qPCR with fluorescence detection. Briefly, the *Quantifiler® Human DNA Quantification* reaction mix (12.5 µL/aliquot) and provided primers (10 µL/aliquot) were placed into the wells of a 96-well plate. Extracted DNA samples (2.5 µL/aliquot) were added to their respective wells to result in a total volume of 25 µL in each well. A standard curve using control DNA supplied by the RCMP (K566, [Promega], 40 ng/µL) was included on every plate. If quantification was not carried out immediately, plates were stored at room temperature in the dark after being sealed with MicroAmp® Optical Adhesive Film [Applied Biosystems]. Otherwise, plates were centrifuged at 4,000 rpm for four minutes to remove bubbles from the wells and were inserted into the 7500 RT-PCR system [Applied Biosystems] to provide readouts correlated to the amounts of human specific DNA in each well based on the standard curve.

DNA amplification and preparation of amplified products

To proceed to the amplification step, the quantity of DNA should be equal or more than 0.250 ng. When samples needed concentrating, Montage™ PCR Centrifugal Filter Devices [Millipore] were used following the protocol outlined in the corresponding user guide, except that an 8 µL volume was used to recover DNA from the membrane rather than 20 µL in order to truly concentrate DNA and obtain maximum DNA quantity for amplification. Samples were diluted or concentrated so that each well contained a total of 0.5 ng of target DNA. To detect the pattern of STR in the DNA samples, the AmpF*l*STR™ Profiler Plus kit was used [Applied Biosystems]. The supplied PCR Reaction Mix (5.7 µL), Profiler Plus Primer Set (3.0 µL) and AmpliTaq Gold™ DNA Polymerase (0.3 µL) were added to each well of the 96-well plate as recommended by the manufacturer. For each

sample, 6 µL of DNA was added for a final reaction volume of 15 µL. Negative controls wells were filled with Filtered Autoclave Distilled (FAD) water and positive controls were filled with a standard DNA (Gm 9947) from the Profiler Plus Kit [Applied Biosystems]. MicroAmp™ Amplification Adhesive Film [Applied Biosystems] was secured over the wells and the plates were placed in thermocyclers MJ Research PTC-200 [*St. Bruno, Québec, Canada*] or Bio-Rad C1000 [*Mississauga, Ontario, Canada*]) and covered with a jelly mat. Amplification was completed using the following cycling parameters: 95 ºC, eleven minutes followed with 28 cycles of denaturation for 60 seconds at 94 ºC, annealing of primers for 90 seconds at 59 ºC and extension for 90 seconds at 72 ºC. A final extension at 60 ºC for 75 minutes followed by an overnight incubation at room temperature was also included. The 96-well plates were stored at -20 ºC until ready to be analyzed using CE.

Amplified products were prepared robotically for analysis on the 3100 CE unit using the Tecan Freedom EVO® liquid handling workstation (Tecan Group Ltd., [*Männedorf, Switzerland*]). Plates were centrifuged at 4,000 rpm for five minutes. HiDi Formamide (20 µL) and the internal standard GeneScan™ 500 ROX™ (0.5 µL) [Applied Biosystems] were loaded into each well of a 96-well CE plate and 0.25 µL of appropriate amplified product was added. AmpF*l*STR™ Profiler Plus Allelic Ladders (0.75 µL) [Applied Biosystems] were included on each plate. Prior to each run, the tips of the liquid handling system were cleaned with 70 % ethanol and a pipetting test was performed to ensure that the instrument was dispensing accurately. CE plates were heated at 95 ºC for five minutes and cooled at 4 ºC for three minutes, centrifuged, then loaded onto the CE 3130X Genetic Analysis instrument [Applied Biosystems] following RCMP procedures to derive the sizes of the amplified products. After each run, the instrument tips were cleaned with bleach solution (2 % sodium hypochlorite).

Interpretation of capillary electrophoresis results

Results were interpreted by RCMP NDDB DNA Analyst, Peggy Philion, using Genescan v.3.7 and Genotyper® v.3.7 software packages [Applied Biosystems]. The GeneScan™ 500 ROX™ internal standards were used to create a standard curve from which was extrapolated the size of each amplified STR fragments. Genotyper converts estimated

size into allele designation using AmpF*l*STR™ Profiler Plus Allelic Ladders. The electropherograms produced by the CE were analysed to determine the number of repeats in the selected STR from the amplified human DNA. Nine STR loci on various regions of human chromosomes are routinely included in analysis at the RCMP: D3S1358, vWa, FGA, D8S1179, D21S11, D18S51, D5S818 and D13S317 and D7S820 (Table 2). The gender determining region (amelogenin) is included to determine the gender of the donor of the biological sample. The quality of the DNA profiles was assessed by examining the slope of the profiles, which is indicative of DNA degradation or PCR inhibition. Slope was determined by dividing the area of the peak (homozygous) or peak averages (heterozygous) of the shortest STR locus (D3S1358) to the largest STR locus (D18S51). A positive DNA control, provided in the AmpF*l*STR™ Profiler Plus kit [Applied Biosystems], and a negative control were included for each analysed CE plate.

Statistical Analysis

For each experiment, various volumes of blood (0.1 µl, 0.5 µL and 1.0 µL) from two donors were run in parallel, each divided into non-contaminated and contaminated samples. The entire set was run in duplicate. Buccal swabs were collected from one donor and were divided into contaminated and non-contaminated samples and were prepared in quadruplicate. Every experiment was repeated at least three times. Results from all three experiments were pooled for statistical analysis, performed with Microsoft Office Excel 2007 software. Standard deviations (STDEV) were calculated for each set of extraction samples. A two tail paired Student T-test was performed to compare results from the different extraction methods. If the p-value was below 0.05, the variations between samples were considered significant, and if the p-value was above 0.05 the variations between samples were not considered significant.

CHAPTER III

RESULTS

The overall goal of the project was to further characterize the proposed methodology for removal or inactivation of bacteria and spores from biological human samples. This included testing of the protocol to remove toxins contamination in biological samples without compromising the quantity and quality of human DNA.

Preliminary experiments

The project was designed to include the comparison of DNA extraction using kits, designated as manual and automated, from two different manufacturers. In both cases, all reagents for DNA extraction are provided and magnetic beads are used to isolate DNA. It was suggested that samples in early stage of the process could be harmonized to limit the number of samples. Currently, the RCMP uses a specific buffer for the lysis of cells contained in collected biological samples using a variant of the manual system. Consequently, it was decided to test the performance of this buffer with the automated system. The experiment consisted of comparing the quantity and quality of extracted DNA with the automated protocol using the RCMP lysis buffer or the buffer provided with the automated kit, called G2 [Qiagen]. As a control, DNA was extracted using the manual protocol [Promega] using only the RCMP lysis buffer. This buffer was prepared by mixing 100 mM NaCl, 10 mM Tris HCl (pH 8.0), 10 mM EDTA, 0.5 % sarkosyl and 40 mM DTT. The G2 buffer formulation was 800 mM Guanidium chloride-HCl, 30 mM Tris-HCl (pH 8.0), 30 mM EDTA (pH 8.0), 5 % Tween-20 and 5 % Triton-X100. Proteinase K (8.7 µL) was added to both buffers to result in a final concentration of 1.2 mg/mL.

DNA extraction was performed in two trials, as a preliminary experiment, from varying volumes of blood (0.01 µL to 50 µL) applied onto cotton swabs and buccal swabs obtained from two different donors, and was set up to have parallel duplicate samples for each extraction method. All samples were suspended in the desired lysis buffer with proteinase K and incubated overnight at 56 ºC, prior to DNA extraction.

The resulting DNA samples were quantified and the success of STR analysis was used as a measure of DNA integrity. Figure 3 shows the standard curve of DNA quantity used to calculate the amount of DNA in samples. A slope of -3.3 indicates 100 % PCR

amplification efficiency for the assay, and the R^2 value measures the closeness of fit between the standard curve regression line and the data points, with 1.00 being a perfect fit. The mean amounts of DNA isolated from four samples using different buffers with the automated system are shown in Table 3A. For all volumes of blood and buccal swabs, except for 0.01 µL blood with the G2 buffer, the automated system yielded amounts of human DNA equal or above the threshold of 0.250 ng (minimum amount needed to proceed with analysis), regardless of the buffer used. Surprisingly, the amount of DNA was greater for samples processed with the RCMP buffer, compared to the G2 buffer, with the exception of the smallest volume of blood (0.1 µL) and buccal swabs. No statistical differences were observed between samples extracted with either buffer. All samples were processed for STR profile development. When analyzing the success of STR detection with DNA isolated by the robot in either buffer, the DNA purified with the RCMP lysis buffer showed better profile results (22 out of 24 samples), compared to the G2 buffer (20 out of 24 samples). The results are displayed as the number of samples yielding a complete STR profile out of the sample size of 4 (Table 3A, column labelled completed STR profiles). Based on the DNA extraction results, it was decided to use the RCMP lysis buffer, which will be henceforth referred to as lysis buffer, in all experiments.

In these preliminary experiments, comparison of the manual extraction to automated DNA extraction indicated that the amounts of DNA yielded by the latter were greater for all samples (Table 3B). Significant statistical differences were observed for all types of samples. The automated process also resulted in less degradation of DNA, as shown by fewer partial profiles numbers and a greater number of completed profiles, 22 out of 24 samples, compared to the manual extraction where only 13 out of 24 samples were successful. This difference might be due to variations in the concentration of magnetic beads that capture the DNA in the two kits, but also could be due to a lack of accuracy in the manual kit, which is dependent on the skill of the person carrying out the work.

It was noted during these preliminary experiments that the 10 and 50 µL blood volumes were too concentrated and required significant dilution to achieve DNA collection during amplification. The same was needed for the buccal swab samples. It was also found

that the volume of 0.01 µL yielded inconsistent results, with DNA quantities that were generally close to or below the RCMP threshold of 0.250 ng. For this reason, the standard volumes of blood used in all further experiments were 0.1 µL, 0.5 µL and 1 µL. Buccal swabs were kept as part of the analysis to diversify the type of DNA samples, even though the results were inconsistent.

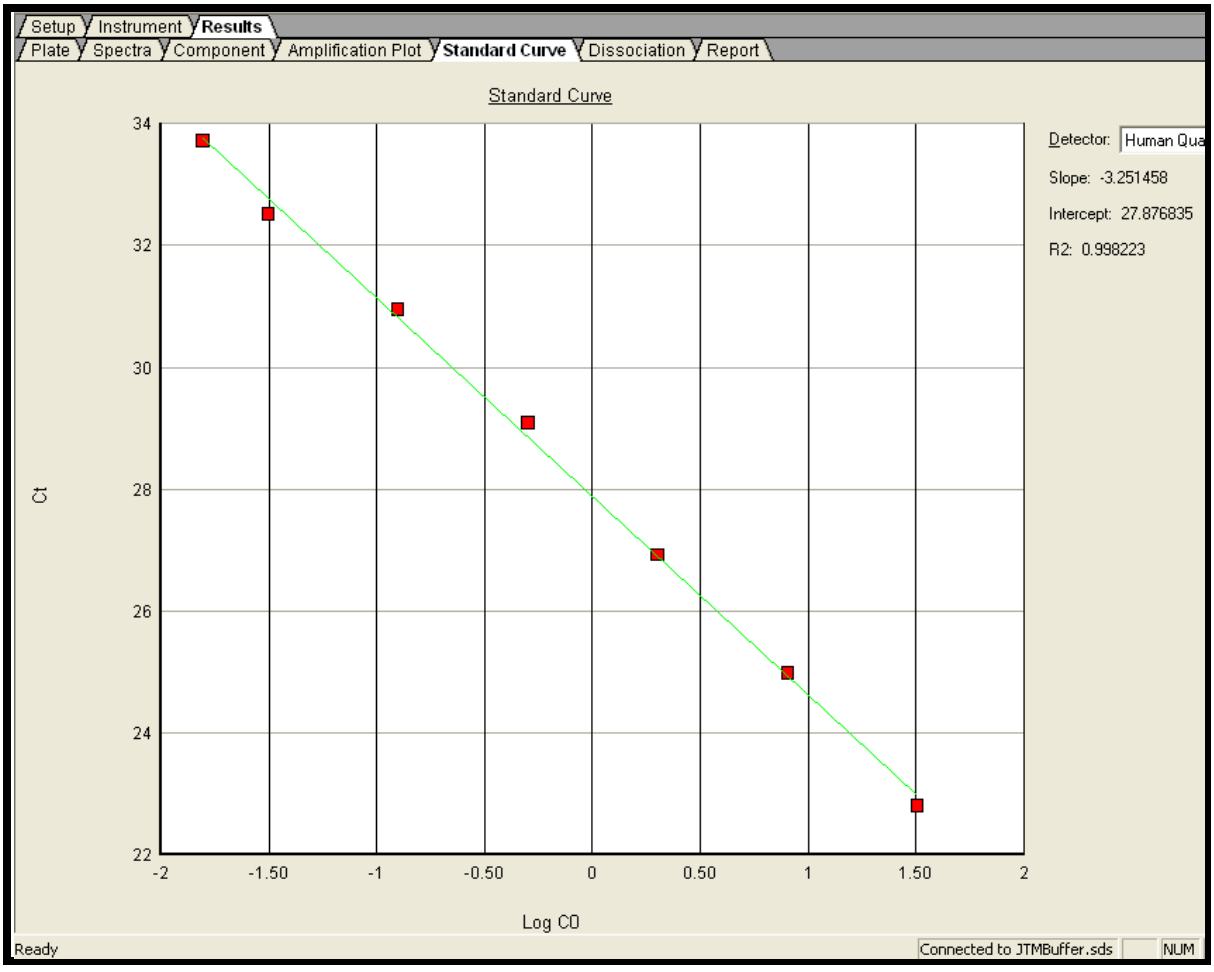

FIGURE 3 **Standard curve for DNA quantification. C_T: threshold cycle of a specified amplification plot that occurs when the fluorescent signal increases beyond the value of the threshold setting. DNA concentration ranged from 32 ng/µL to 0.0156 ng/µL.**

TABLE 3 **Effect of various lysis buffers (RCMP and G2) containing proteinase K on amounts of DNA isolated (ng) and amplification success using manual and automated DNA extraction methods.**

A) Comparison of DNA extraction using two buffers (RCMP and G2) plus the addition of proteinase K (1.2 mg/mL) with the automated protocol.

	AUTOMATED			AUTOMATED		
Sample	**Buffer**	**Average DNA quantity (ng ± STDEV)**	**Completed STR profiles**	**Buffer**	**Average DNA quantity (ng ± STDEV)**	**Completed STR profiles**
Blood (50 µL)	G2	218.1 ± 126.9	4/4	RCMP	488.9 ± 77.1	4/4
Blood (10 µL)	G2	53.7 ± 11.4	4/4	RCMP	90.4 ± 10.8	4/4
Blood (1 µL)	G2	4.18 ± 1.50	3/4	RCMP	7.38 ± 4.63	4/4
Blood (0.1 µL)	G2	0.65 ± 0.18	3/4	RCMP	1.75 ± 0.23	4/4
Blood (0.01 µL)	G2	**0.20 ± 0.10**	2/4	RCMP	0.25 ± 0.08	2/4
Buccal swab (unknown)	G2	257.9 ± 108.0	4/4	RCMP	186.8 ± 108.9	4/4

Bold red = amount below the threshold of 0.250 ng; p = partial profile. (n=4).

B) Comparison of two extraction methods (manual and automated) with the chosen lysis buffer (RCMP).

	MANUAL			AUTOMATED		
Sample	**Buffer**	**Average DNA quantity (ng)**	**Completed STR profiles**	**Buffer**	**Average DNA quantity (ng)**	**Completed STR profiles**
Blood (50 µL)	RCMP	3.70 ± 0.58	3/4 (1p)	RCMP	488.9 ± 77.1	4/4
Blood (10 µL)	RCMP	0.70 ± 0.68	3/4	RCMP	90.4 ± 10.8	4/4
Blood (1 µL)	RCMP	0.38 ± 0.28	4/4	RCMP	7.38 ± 4.63	4/4
Blood (0.1 µL)	RCMP	**0.23 ± 0.03**	0/4 (1p)	RCMP	1.75 ± 0.23	4/4
Blood (0.01 µL)	RCMP	**0.15 ± 0.10**	1/4	RCMP	0.25 ± 0.08	2/4
Buccal swab unknown)	RCMP	13.3 ± 16.8	2/4 (2p)	RCMP	186.8 ± 108.9	4/4

Bold red = amount below the threshold of 0.250 ng; p = partial profile. (n=4).

Establishment of extraction method

Based on the preliminary experiments, the following protocol was generated for subsequent experiments (Figure 4). Blood samples from two donors were diluted to produce samples of 0.1 µL (10 µL of 1:100 blood dilution), 0.5 µL (10 µL of 1:20 blood dilution) and 1 µL (10 µL of 1:10 blood dilution). Duplicate samples from each donor were placed directly on a sterile cotton swab and left to air-dry on a sterile Petri dish with a DNA contamination-free surface for an hour, resulting in a total of twelve samples. As buccal swabs were collected from only one donor, four rather than two replicates of a single dilution were generated. For each set of experiments, there were non-contaminated samples and contaminated samples, established with the addition of 100 µL of the bacterial agent or 10 µL of a toxin prior to the drying step. In all experiments, the following controls were included: two negative controls (blank swabs), a swab with the selected biological agent but no human sample, and whole blood swabs without contamination. After drying, swabs were placed in sterile tubes, and lysis buffer (341.3 µL) and 50 mg/mL of Proteinase K (8.7 µL) were added. Tubes were incubated overnight at 56 ºC, and then the lysate sample was separated from the swab by centrifugation (9,300 xg, 5 minutes) through a spin basket. The swab was then discarded and 1 µg/µL cRNA was added to each lysate. Equal volumes (150 µL) were then processed by the manual or automated method in most experiments. Finally, DNA eluates were passed through a 0.22 µm filter by centrifugation prior to quantification and STR profile development as described (Hause, 2007).

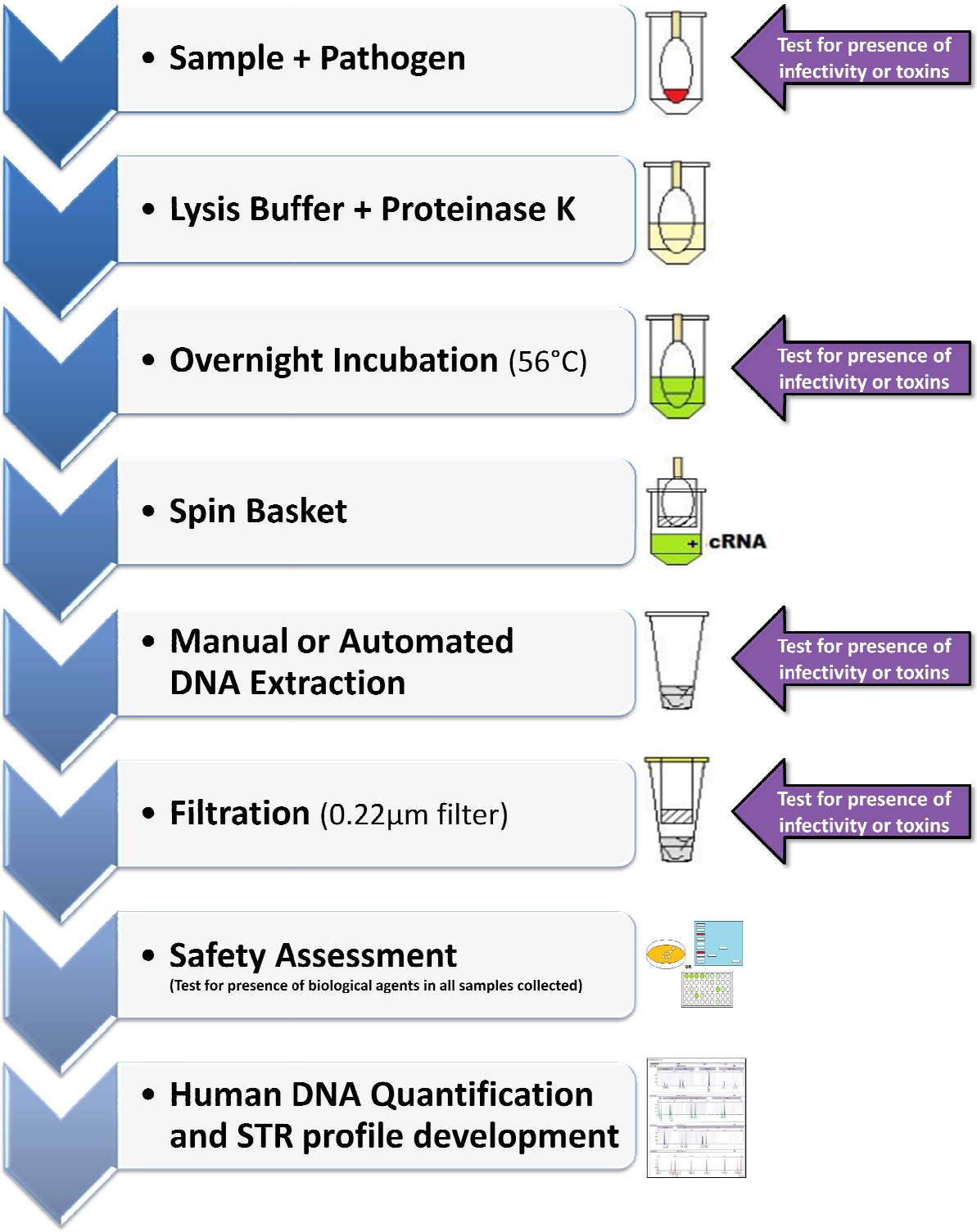

FIGURE 4 **Proposed methodologies for contaminated DNA extraction and purification using modified protocols from manual or automated DNA extraction.**

Confirmation of loss of infectivity

Human DNA samples must be free of material that has the potential to be harmful to forensic analysts in order to be processed in a forensic laboratory. The first objective of this project was to confirm whether the preliminary protocol developed for the removal of infectious bacteria and spores by the addition of a filtration step after the extraction of DNA could eliminate or inactivate selected infectious material. It had been shown that the integration of a filtration step to the DNA extraction procedure currently used in forensic laboratories yielded safe DNA suitable for human profiling (Hause, 2007). However, it was not established whether the filter was really the cause of the removal of the selected bacteria, which were *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus anthracis*, *Yersinia pestis* and *Francisella tularensis*. The completed experiments showed that incubation at temperatures as high as 95 ºC could not guarantee complete inactivation of bacteria and spores, but it is unclear if the other steps in the DNA extraction process could reduce or remove the presence of these pathogens.

The various steps in the DNA extraction process are designed to denature and eliminate proteins. Hence, it seemed likely that infectivity of bacteria could be lost throughout the procedure. To test this, experiments were prepared as described below with the intention to determine at which step of the extraction the infectivity was reduced or lost. For these experiments, 1 µL blood samples were prepared on cotton swabs, as previously described, and then 10^9 bacteria or 10^9 spores were added to the swabs and were dried. This amount was chosen to test a higher concentration of bacteria than had previously been used (Hause, 2007). In addition, higher numbers of spores or bacteria may be mimic what might be expected in a situation where large amounts of bacteria or spores were being manufactured in a bioterrorist's laboratory. For vegetative bacteria, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Yersinia pseudotuberculosis* were chosen as representative Gram-negative and Gram-positive bacteria. *Yersinia pseudotuberculosis* was used as a surrogate for *Yersinia pestis*. For the examination of bacterial spores, two strains of *Bacillus anthracis* were used. The Sterne strain is of low virulence, while the bovine strain is highly virulent.

It was necessary to prepare stocks to be used for seeding blood and buccal samples. Growth curves for vegetative bacteria were established which would allow for the assessment of infectious CFU from the OD measurement at 600 nm. For this purpose, the OD of samples taken at hourly intervals was plotted against CFU of the same samples to generate a growth curve for a future seeding use. The results are shown in Figure 5 for *Staphylococcus aureus*, in Figure 6 for *Pseudomonas aeruginosa* and in Figure 7 for *Yersinia pseudotuberculosis*. The amount of vegetative bacteria added to the sample swabs was calculated using these growth curves to obtain an amount of 10^9 bacteria per sample.

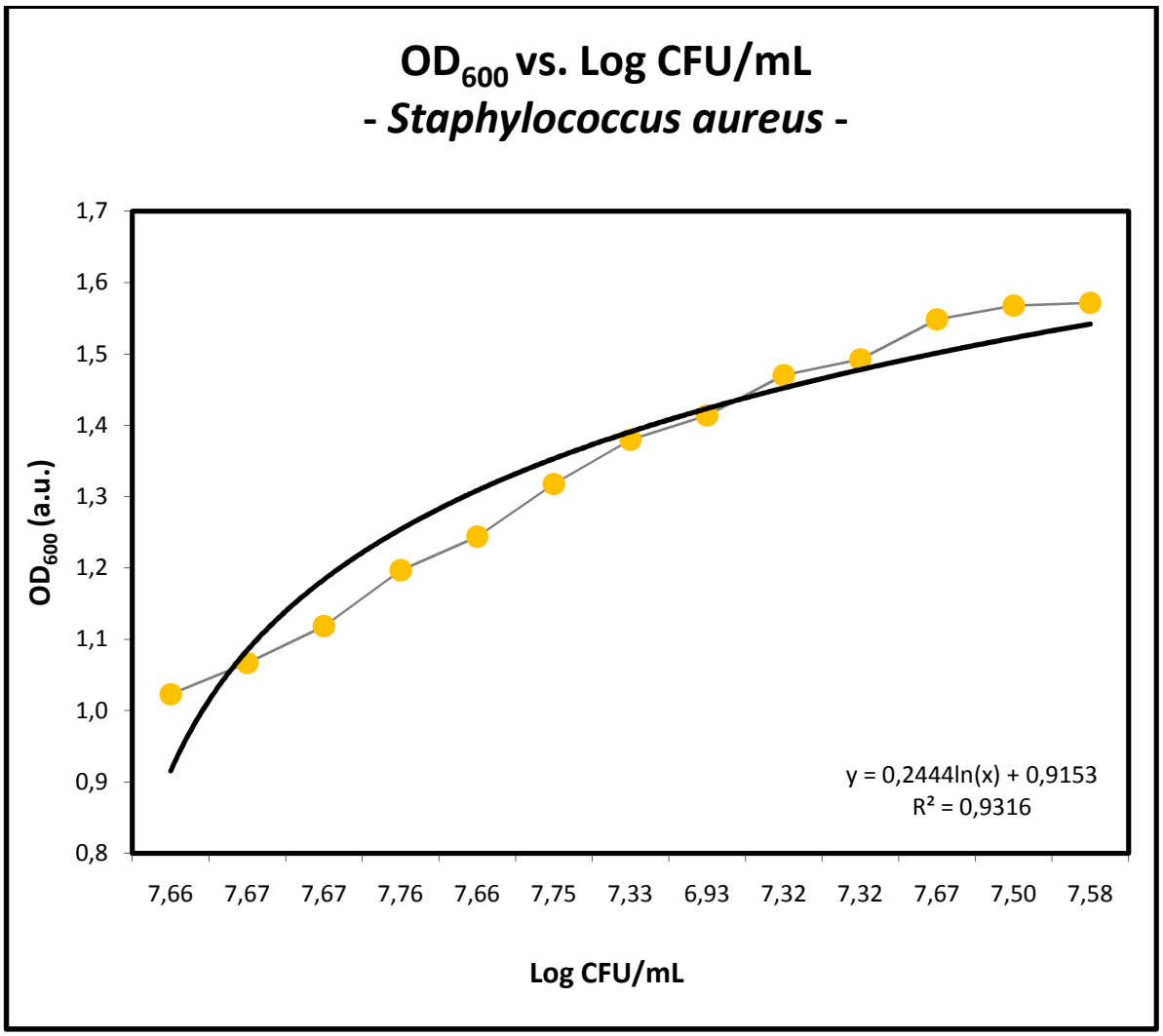

FIGURE 5 **Growth of *Staphylococcus aureus* during the first 48 hours of culture of in tryptic soy broth at 37 ºC. (n=3). Doted line = absorbance values (OD_{600}) base on the number of counted colonies in a logarithmic format (Log CFU/mL); Black line = exponential growth.**

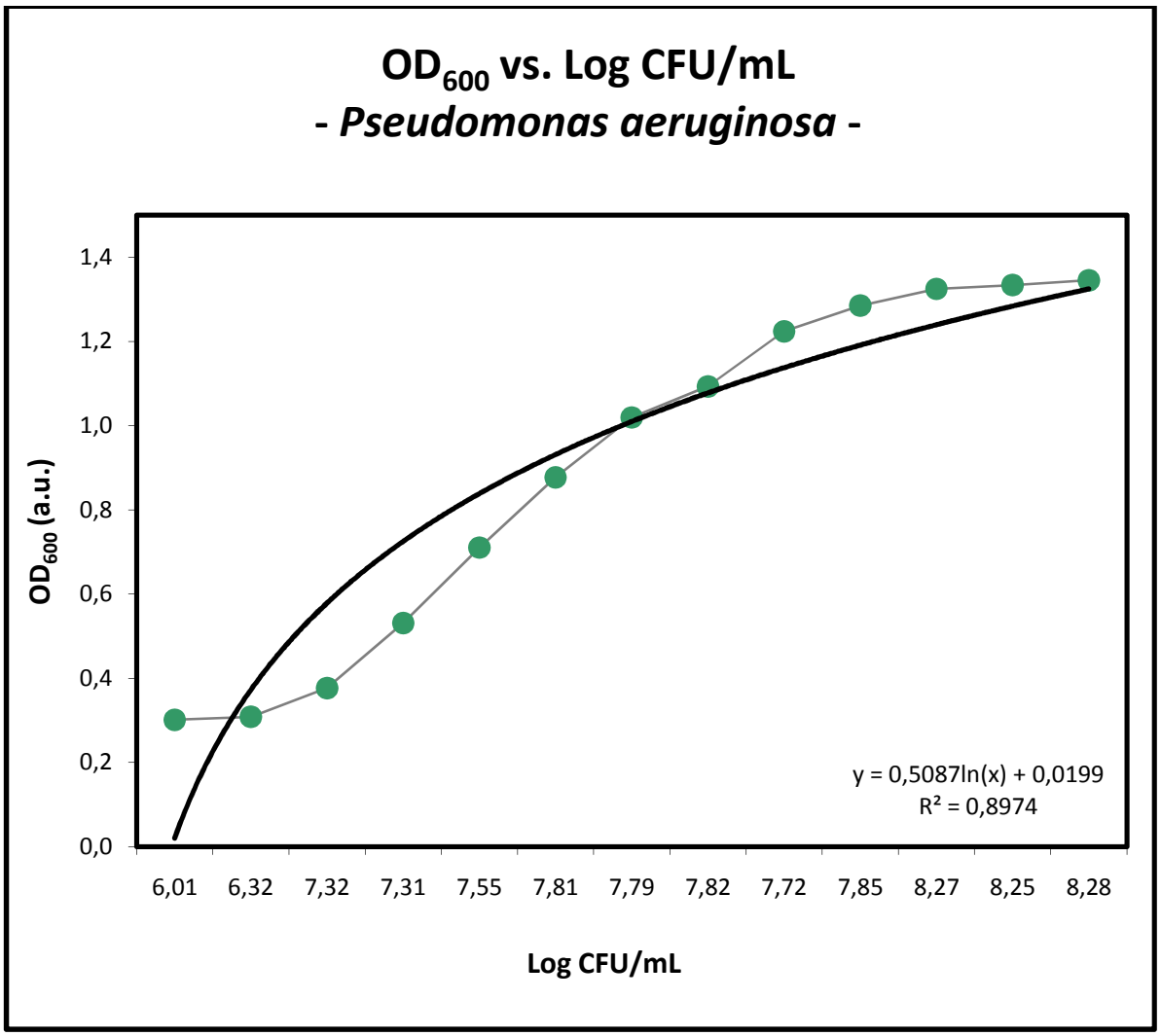

FIGURE 6 **Growth of *Pseudomonas aeruginosa* during the first 48 hours of culture in tryptic soy broth at 37 ºC. (n=3). Doted line = absorbance values (OD_{600}) base on the number of counted colonies in a logarithmic format (Log CFU/mL); Black line = exponential growth.**

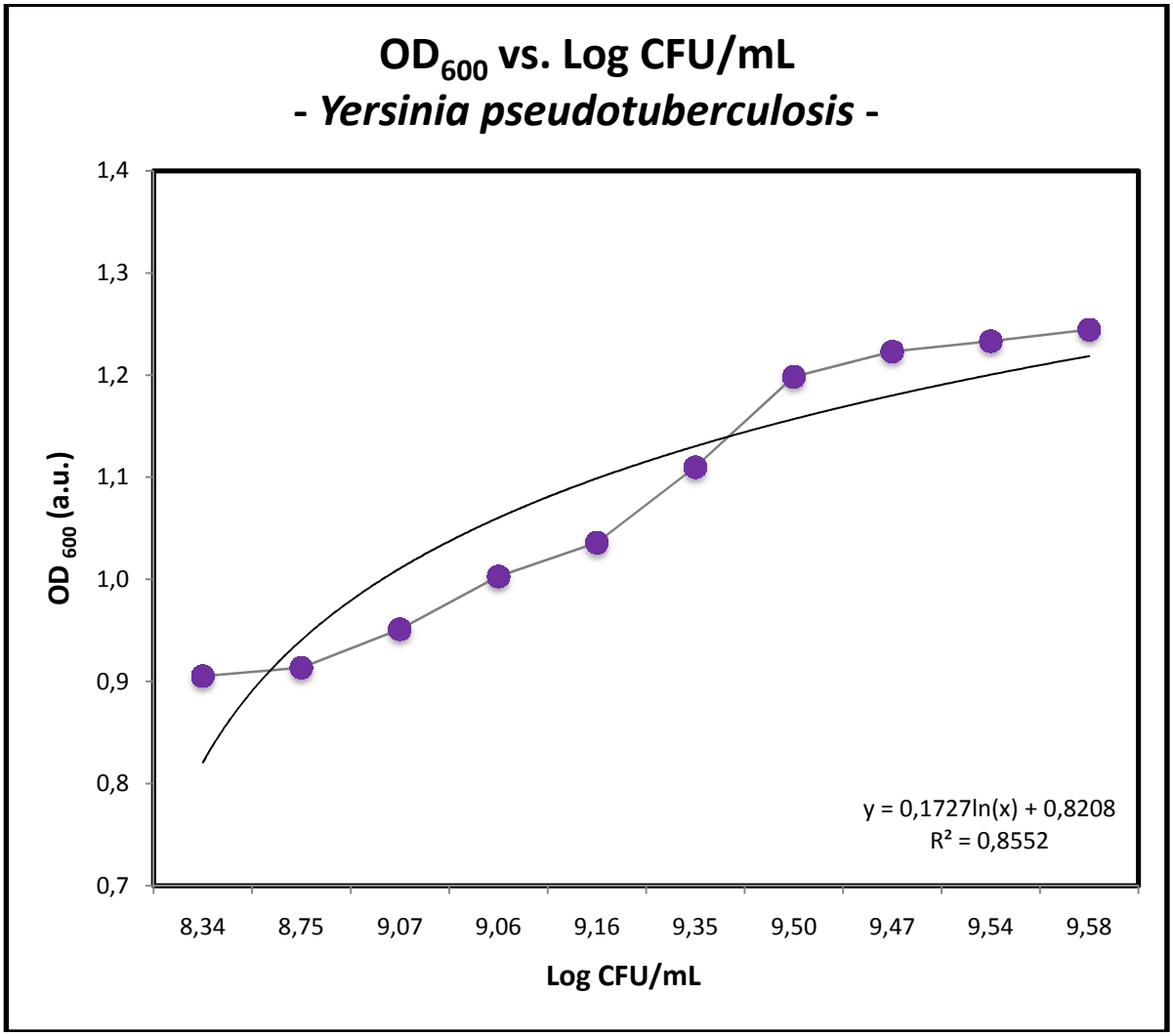

FIGURE 7 **Growth of *Yersinia pseudotuberculosis* during the first 72 hours of culture in tryptic soy broth at 37 ºC. (n=3). Dotted line = absorbance values (OD_{600}) base on the number of counted colonies in a logarithmic format (Log CFU/mL); Black line = exponential growth.**

To determine at which stage(s) of the DNA extraction protocol the samples were safe for processing, whole samples (100 µL) were tested for the presence of the contaminant at the following stages: 1) after the overnight lysis at 56 ºC in buffer with proteinase K; 2) at the end of the DNA extraction but before filtration; 3) at the end of the DNA extraction but after filtration. In addition, swabs were tested after incubation at 56 ºC in PBS (without lysis buffer), when filtration was carried out prior to DNA extraction, and when filters were compromised.

As can been seen in Table 4, all three bacteria were readily inactivated during the first step of the extraction process, which was an overnight incubation at 56 ºC in lysis buffer plus proteinase K. Furthermore, it was revealed that the overnight incubation at 56 ºC was enough to eliminate infectious *Staphylococcus aureus* and *Pseudomonas aeruginosa* from samples, but was not enough for one sample contaminated with *Yersinia pseudotuberculosis*. For samples that underwent the DNA extraction with and without filtration, no growth was observed out of the eighteen samples following DNA capture. In previous experiments, filtration before incubation at 56 ºC failed to remove infectious material from one out of nine samples (Hause, 2007). However, subsequent examination of the filter indicated that the filter had been damaged during pipetting (Hause, 2007). Indeed, deliberate damage to the filter before incubation resulted in growth of all samples (9 samples).

TABLE 4 **Loss of infectivity of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Yersinia pseudotuberculosis* at various stages of the DNA extraction process.**

	Staphylococcus aureus* (CFU)	***Pseudomonas aeruginosa* (CFU)***	***Yersinia pseudotuberculosis* (CFU)***
Positive growth control	≥150 / ≥150 / ≥150	≥150 / ≥150 / ≥150	≥150 / ≥150 / ≥150
After O/N freeze (aliquot)	≥150 / ≥150 / ≥150	≥150 / ≥150 / ≥150	≥150 / ≥150 / ≥150
O/N incubation at 56 °C	0 / 0 / 0	0 / 0 / 0	4 / 0 / 0
O/N cell lysis at 56 °C	0 / 0 / 0	0 / 0 / 0	0 / 0 / 0
DNA capture	0 / 0 / 0	0 / 0 / 0	0 / 0 / 0
After filtration	0 / 0 / 0	0 / 0 / 0	0 / 0 / 0
Filtration before incubation	0 / 0 / 0	0 / 0 / 0	≥ 150** / 0 / 0
Tampered filter before incubation	145 / 133 / 139	142 / 142 / ≥150	≥150 / 146 / ≥150

0 = no growth; O/N = overnight. (n=3).
***Assessed by plating aliquots in triplicate on TSA plates after indicated treatment.**
****** **Filter was damaged during pipetting.**

Spores were expected to be more resistant to heat and the lysis buffers, based on the known resistance of spores to extreme conditions, and from previous work (Hause, 2007). Spores from two *Bacillus anthracis* strains were applied to human biological samples in order to obtain 10^9 spores per sample, and infectivity was assessed as in the previous experiments. The results in Table 5 show that an incubation at 56 ºC alone had a small effect on the number of infectious spores, but an incubation in the presence of the lysis buffer with proteinase K reduced the number of spores present by approximately four-fold when compared with heat alone. A further growth reduction of approximately ten-fold was observed for samples that underwent the complete DNA capture. However, growth of *Bacillus anthracis* was still observed in all twelve samples after the extraction process. No growth was observed before and after filtration in the twelve samples exposed to both strains of *Bacillus anthracis*. Therefore, the final filtration step was necessary to remove all infectious spores in these samples. To prove that spores were captured by the filters, the filters were placed in TSB to allow growth and reveal the presence of infectious *Bacillus anthracis.* Thus, it was concluded that when forensic samples are contaminated with unknown infectious agents, the filtration step is essential to ensure the elimination of spores prior to the analysis of the DNA. The filtration step was kept for all subsequent experiments.

TABLE 5 **Loss of infectivity of *Bacillus anthracis* spores at various stages of the DNA extraction process.**

	Bacillus anthracis* *Sterne strain* (CFU)	***Bacillus anthracis* *NML 03-0191* (CFU)***
Positive growth control	≥150 / ≥150 / ≥150	≥150 / ≥150 / ≥150
After O/N freeze (aliquot)	ND	≥150 / ≥150 / ≥150
O/N incubation at 56 °C	ND	124 / 109 / 131
O/N cell lysis at 56 °C	45 / 34 / 37	31 / 42 / 32
DNA capture	4 / 4 / 3	3 / 4 / 3
After filtration	0 / 0 / 0	0 / 0 / 0
Filtration before incubation	ND	0 / 0 / 0
Tampered filter before incubation	ND	≥150 / ≥150 / ≥150

0 = no growth; O/N = overnight; ND = not done. (n=3).
***Assessed by plating aliquots in triplicate on TSA plates after indicated treatment.**

Verification of toxin denaturation

Toxins are biological poisons that generally are small molecules, such as lipids, peptides or proteins. Therefore, the filtration step would not be efficient at trapping the toxins. However, as toxins are mostly proteinaceous or lipid in nature, the incorporation of detergent (SDS, DTT and sarkosyl) and proteinase K in the lysis buffer should inactivate or degrade toxins prior to DNA extraction. To confirm this, the presence of various toxins seeded into human samples was verified at various stages during the DNA extraction process. For these experiments, the approach was to aliquot blood samples of 1 µL onto three cotton swabs for samples to be treated with heat alone (56 ºC) or with lysis buffer, proteinase K and heat. Immediately after an hour of air-drying, 10 µL of a designated toxin dose were added to the swab, and dried for an additional hour. Toxins were added to samples at a range of concentrations that would be toxic or lethal to individuals handling the samples, as determined from the LD_{50}. This range varied for each toxin. A set of samples that had been seeded with a single dose of toxin, and have been submitted to the complete DNA capture and filtration were also included.

The first toxin tested was BoNT/A. The LD_{50} for BoNT/A is 0.001 µg/kg when aerosolized, and 1 µg/kg when ingested. For an average person, the dose that would kill 50 % of adults exposed by the aerosolized toxin would be approximately 80 ng, and over 60 µg by ingestion. Blood samples were contaminated with quantities of the toxin ranging from 0.05 to 50 µg. Samples were incubated overnight at 56 ºC in the absence or presence of the lysis buffer plus Proteinase K, then assayed for the presence of the toxin. Because the amount of toxin used in the experiments was as low as 50 ng, BoNT/A was assayed using a commercial ELISA kit capable of detecting quantities as low as 0.1 ng. Six filtered samples that had originally contained 1 µg of the toxin and that had undergone complete DNA capture were also tested for the presence of the toxin.

The absorbance values for samples with various quantities of the toxin are presented in Table 6. The Plus/Minus (+/-) ELISA format uses a positive row of capture antibody above a negative row of normal, same species antibody. The minus result can be subtracted from the plus result to produce a value that should reflect specific reactivity. The cut-off for

positivity in this assay, as suggested by the manufacturer, is determined by adding three times the standard deviation to the mean of the negative control well containing no antigen, then adding 0.150 to this value. The standard curve (toxin alone) was a positive control for the ELISA, and was used to determine the absolute quantity of toxin detected after extraction treatments, as shown in Figure 8.

The amount of detectable BoNT/A in blood decreased in samples incubated at 56 ºC in the presence of detergent and proteinase K below one LD_{50} value, which would not be toxic to anyone. However, the negative controls used to set the threshold for the assay created an unexpectedly high background, and therefore one cannot make clear conclusions about whether or not toxic doses would still be present in the samples.

It was not clear from these observations whether the toxin was degraded during the overnight incubation to the extent that the samples would be safe for analysis. Further extraction steps seemed to demonstrate an additional reduction below the LD_{50} (DNA capture), but again a high background nullified these assumptions. The presence of blood in this assay interfered with the specificity of the ELISA and the detection of BoNT/A. Subsequently, the results obtained in Figure 8 were inconclusive even though the absorbance obtained for some samples was very low and similar to the absorbance obtained in the blanks, which corresponded to the limit of detection of the ELISA kit (1 to 2 ng/mL).

TABLE 6 **ELISA detection of botulinum toxin A in seeded blood samples at various steps in DNA extraction.**

	1	2	3	4	5	6	7	8	9	10	11	12
Toxin added (µg)	**50.0**	**25.0**	**12.5**	**6.25**	**3.13**	**1.56**	**0.78**	**0.39**	**0.19**	**0.10**	**0.05**	**0.00**
	Standard Curve (Toxin alone)											
+	0,320	0,318	0,340	0,322	0,266	0,296	0,260	0,153	0,117	0,094	0,069	0,054
-	0,198	0,184	0,162	0,152	0,233	0,164	0,112	0,078	0,153	0,096	0,067	0,053
Diff.	0,122	0,134	0,178	0,170	0,033	0,132	0,148	0,075	0,000	0,000	0,002	0,001
	Toxin mixed with blood (Untreated)											
+	0,274	0,287	0,261	0,281	0,249	0,207	0,178	0,142	0,118	0,110	0,060	0,021
-	0,369	0,436	0,346	0,394	0,500	0,389	0,383	0,385	0,145	0,131	0,059	0,027
Diff.	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,001	0,000
Toxin added (µg)	**50.0**	**25.0**	**12.5**	**6.25**	**3.13**	**1.56**	**50.0**	**25.0**	**12.5**	**6.25**	**3.13**	**1.56**
	Heat 56 ºC						**Lysis Buffer + Proteinase K + 56 ºC**					
+	0,243	0,297	0,246	0,216	0,241	0,259	0,144	0,179	0,140	0,080	0,030	0,000
-	0,225	0,301	0,238	0,190	0,232	0,338	0,128	0,118	0,114	0,080	0,010	0,000
Diff.	0,018	0,000	0,008	0,026	0,009	0,000	0,016	0,061	0,026	0,000	0,020	0,000
Toxin added (µg)	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
	DNA Capture						**Negative controls**					
+	0,026	0,027	0,013	0,014	0,064	0,055	0,083	0,011	0,074	0,065	0,021	0,011
-	0,088	0,077	0,014	0,012	0,061	0,059	0,081	0,011	0,072	0,098	0,021	0,013
Diff.	0,000	0,000	0,000	0,002	0,003	0,000	0,002	0,000	0,002	0,000	0,000	0,000

Numbers represent OD values at 405 nm. (+) = wells coated with capture antibody; (-) = wells coated with non specific antibody (background); Diff. = difference between the positive row and the negative row; Negative controls = wells with no toxoid added. (n=1).

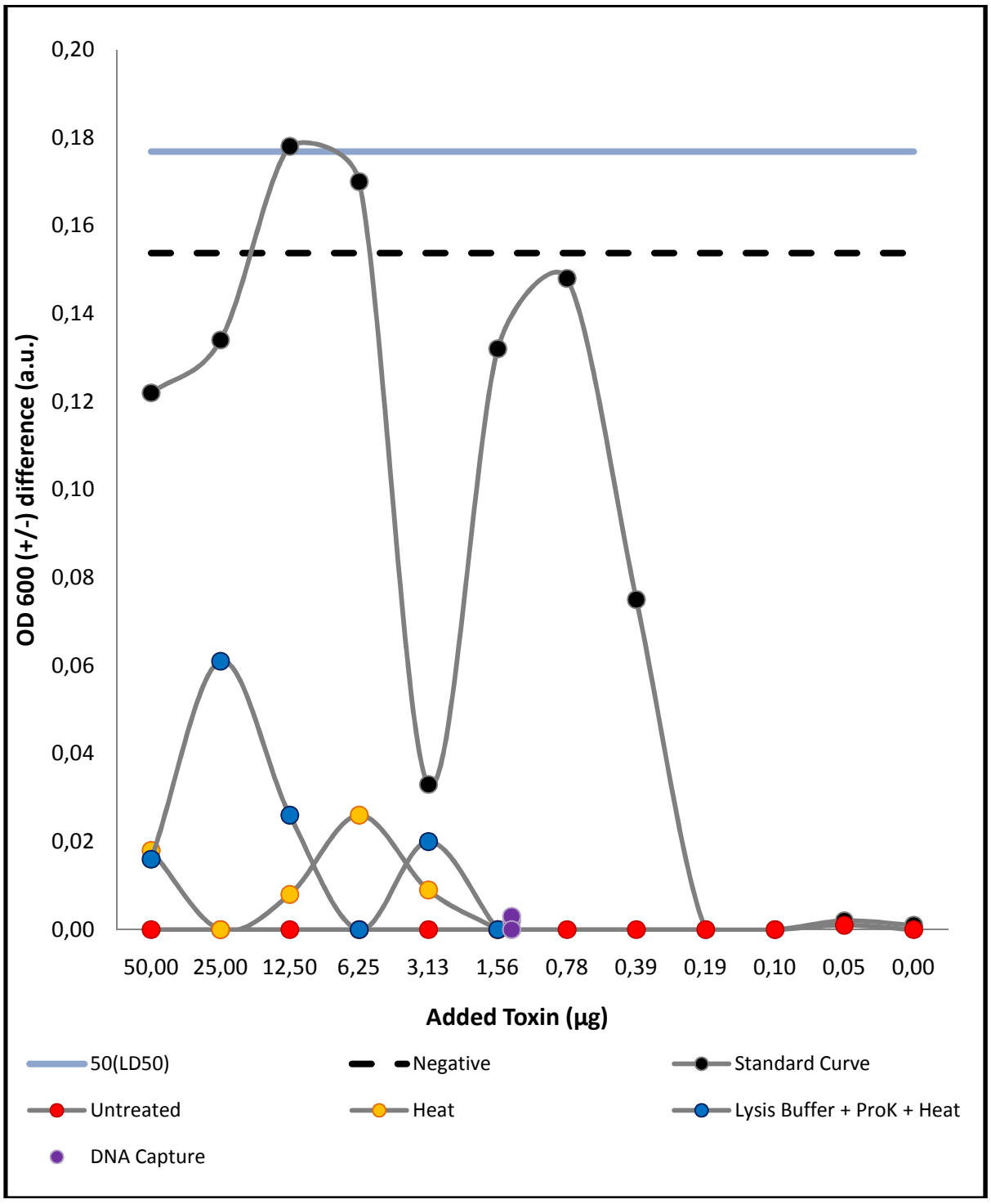

FIGURE 8 **Measured amount of botulinum toxin A based on the standard curve and the absorbance obtained at 405 nm with the spectrophotometer determined by ELISA. Standard Curve = toxin alone; Untreated = Toxin mixed with blood without treatment; Heat = Toxin mixed with blood incubated at 56 ºC alone; Lysis Buffer + proteinase K + Heat = toxin mixed with blood incubated at 56 ºC with lysis buffer and proteinase K; DNA capture = extracted DNA with toxin. (n=1).**

The second toxin in the study was the SEB. The LD_{50} for SEB is 0.03 µg/kg for aerosol exposure (Dinges *et al.,* 2000; Kendall *et al.,* 2008; Roberts *et al.,* 2000), and 1.7 mg/kg for oral ingestion (Rusnak et al., 2004) therefore, doses of SEB ranging from 0.001 to 10 µg were chosen. These larger amounts were likely to be detected by SDS-PAGE, as the detection limit of Coomassie staining is reported to be 50 to 100 ng (EverythingBio.com, 2007). Serial dilutions of the toxin were subjected to a 12.5 % SDS-PAGE, and stained. As shown in Figure 9, which shows results from one of three experiments, only the largest quantities of SEB, 10 µg and 1 µg, were clearly visible at the expected size of 28 kDa although a faint band was present for the 0.1 µg quantity. Amounts of blood used in the previous experiments were mixed with the toxin to observe the effect of detection and degradation after incubation in cell lysis buffer. Blood mixed with toxin showed a smear of proteins, with a large band visible at 28 kDa, which could be the toxin, or a mix of toxin and blood proteins. Incubation in lysis buffer overnight at 56 ºC with proteinase K eliminated all proteins in the samples, with no indication of the continued presence of toxin. The same results were found in all three experiments. Thus, these results show that amounts of SEB as high as 10 µg are degraded during the lysis phase of DNA extraction. Any residual SEB undetected by the Coomassie staining would be below the toxic dose.

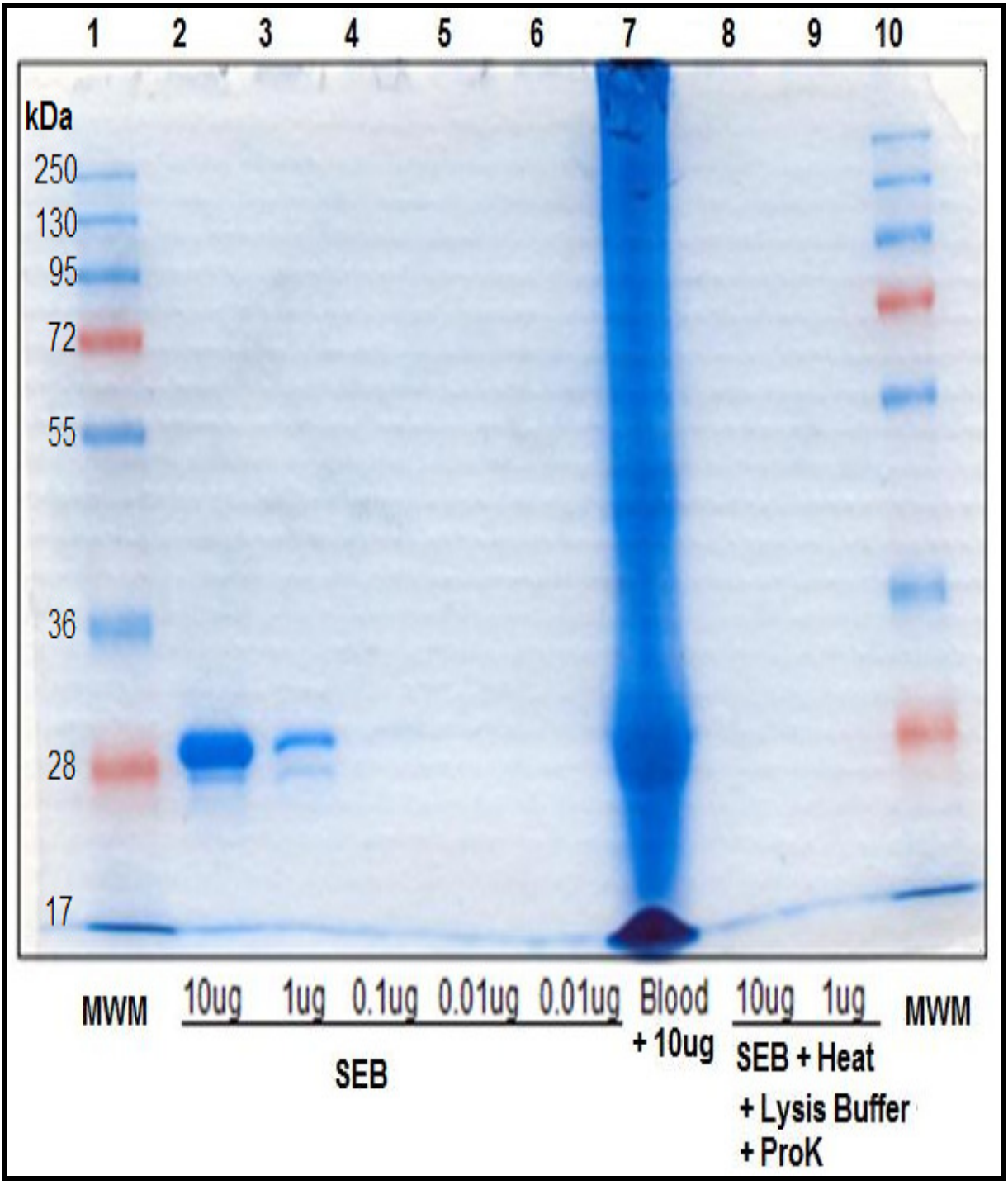

FIGURE 9 **Elimination of staphylococcal enterotoxin B by incubating at 56 ºC in lysis buffer and proteinase K, as detected by Coomassie blue after 12.5 % SDS-PAGE. Sensitivity of Coomassie Blue = 50 to 100 ng; MWM = Molecular weight markers; SEB = toxin alone diluted in sterile water; SEB + Heat = SEB mixed with 10 µL blood, incubated overnight at 56 ºC in buffer as indicated. Results are representative of three experiments (n=3).**

The third toxin that was chosen was ricin. The LD_{50} of ricin for humans when aerosolized is 2.7 µg/kg, which corresponds to a dose of approximately 216 µg for an average person of 80 kg. Because of the restriction in acquisition of this toxin, it was decided to substitute a ricin toxoid in these experiments. As before, blood samples were contaminated with varying quantities of the ricin toxoid ranging from 0.1 to 100.0 µg. The amount of toxoid available for these experiments was limited, and the toxoid quantities added to the samples are below the lethal dose. At various steps, samples were tested for the presence of the toxoid, including samples seeded with 3 µg of toxoid, based on LD_{50}, which underwent the full DNA capture process. An ELISA kit was available for the detection of ricin. As the toxoid had only two amino acid differences in the linker compared to ricin, it was expected that this kit would also detect the toxoid. Like the ELISA for BoNT/A, this is a capture kit with control wells that have a non-specific antibody alternating with rows containing a ricin-specific antibody. Ricin specific reactivity is determined by subtracting the OD values of the negative wells from the positive wells. The threshold for positivity was determined to be an absorbance of 0.513 as described above.

As shown in Table 7 that for the standard curve of toxin alone, or toxin in the presence of blood, there was ricin specific reactivity, as indicated by the OD values in the rows labelled as "Difference". Based on the standard curve of toxoid in blood, the limit of detection of the assay is below 0.1 µg. As revealed in Figure 10, the incubation at 56 ºC alone or with the lysis buffer with proteinase K degraded the toxoid to levels below the LD_{50}. During the lysis treatment of the toxoid, the absorbance of toxoid was lowered from the quantity measured at 100 µg to the absorbance measured at 0.4 µg (12 samples) (Table 7). Similar results were observed with heat only (12 samples). Based on the literature, prevention of toxicity of ricin can be achieved by inactivating the toxin at 70 ºC; the temperature at which the ricin protein irreversibly unfolds (Barnes *et al.,* 2009; CDC, 2010). Since this is a recombinant ricin toxoid containing extra amino acids in the linker between the two subunits, the temperature needed to inactivate the protein could have been lowered. It is important to note that a simple overnight heat treatment at 56 ºC in sterile water might not inactivate the actual toxin. Additional steps in the DNA extraction process did not further reduce the amounts of toxoid. The decrease in absorbance from some of the samples could

be explained by the fact that during the process of automation of DNA isolation, some of the lysate was left behind, along with the toxic protein. The absorbance was high compared to the proposed limit of detection of the commercial ELISA kit for ricin (30 to 40 pg).

Based on the literature, degradation by the detergent and the proteinase K during an overnight incubation at 56 ºC has been shown to inactivate the toxicity of the studied proteins (Dale & von Schantz, 2007). Indeed, proteinase K is a broad-spectrum serine protease with a carboxyl group of aliphatic and aromatic amino acid as its predominant site of cleavage (Qiagen, 2009). Therefore, the DNA extraction methodology does not need to be modified to deal with the presence of toxins.

TABLE 7 **ELISA detection of recombinant ricin toxoid in seeded blood samples at various steps in DNA extraction.**

	1	2	3	4	5	6	7	8	9	10	11	12
Toxoid added (µg)	**100.0**	**50.0**	**25.0**	**12.5**	**6.3**	**3.1**	**1.6**	**0.8**	**0.4**	**0.2**	**0.1**	**0.0**
	Standard Curve (Toxoid alone)											
+	2,645	2,660	2,590	2,530	2,860	2,570	2,330	2,480	1,600	0,790	0,630	0,422
-	0,753	0,730	0,870	0,990	0,470	0,440	1,000	1,470	1,450	0,630	0,080	0,406
Diff.	1,892	1,930	1,720	1,540	2,390	2,130	1,330	1,010	0,150	0,160	0,550	0,016
	Toxoid mixed with blood (Untreated)											
+	2,406	3,040	2,690	2,600	2,620	2,200	2,040	2,750	2,080	1,310	0,570	0,300
-	1,553	1,450	1,350	1,520	1,630	1,480	1,500	1,270	1,250	0,740	0,690	0,119
Diff.	0,853	1,590	1,340	1,080	0,990	0,720	0,540	1,480	0,830	0,570	0,000	0,181
Toxoid added (µg)	**100.0**	**50.0**	**25.0**	**12.5**	**6.3**	**3.1**	**100.0**	**50.0**	**25.0**	**12.5**	**6.3**	**3.1**
	Heat 56 ºC						**Lysis Buffer + Proteinase K + 56 ºC**					
+	0,956	1,460	1,250	1,100	1,390	1,150	1,090	1,110	1,060	0,360	0,170	0,242
-	0,761	0,920	1,170	1,180	1,160	1,260	1,250	0,880	1,350	0,120	0,230	0,143
Diff.	0,195	0,540	0,080	0,000	0,230	0,000	0,000	0,230	0,000	0,240	0,000	0,099
Toxoid added (µg)	**3.0**	**3.0**	**3.0**	**3.0**	**3.0**	**3.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
	DNA Capture						**Negative controls**					
+	0,938	0,500	0,930	1,100	1,350	1,070	1,090	1,280	1,010	1,050	0,340	0,799
-	0,760	0,370	0,780	1,010	0,950	0,910	0,900	1,020	0,850	0,866	0,280	0,645
Diff.	0,178	0,130	0,150	0,090	0,400	0,160	0,190	0,260	0,160	0,184	0,060	0,154

Numbers represent OD values at 405 nm. (+) = wells coated with capture antibody; (-) = wells coated with non specific antibody (background); Diff. = difference between the positive row and the negative row; Negative controls = wells with no toxoid added. (n=1).

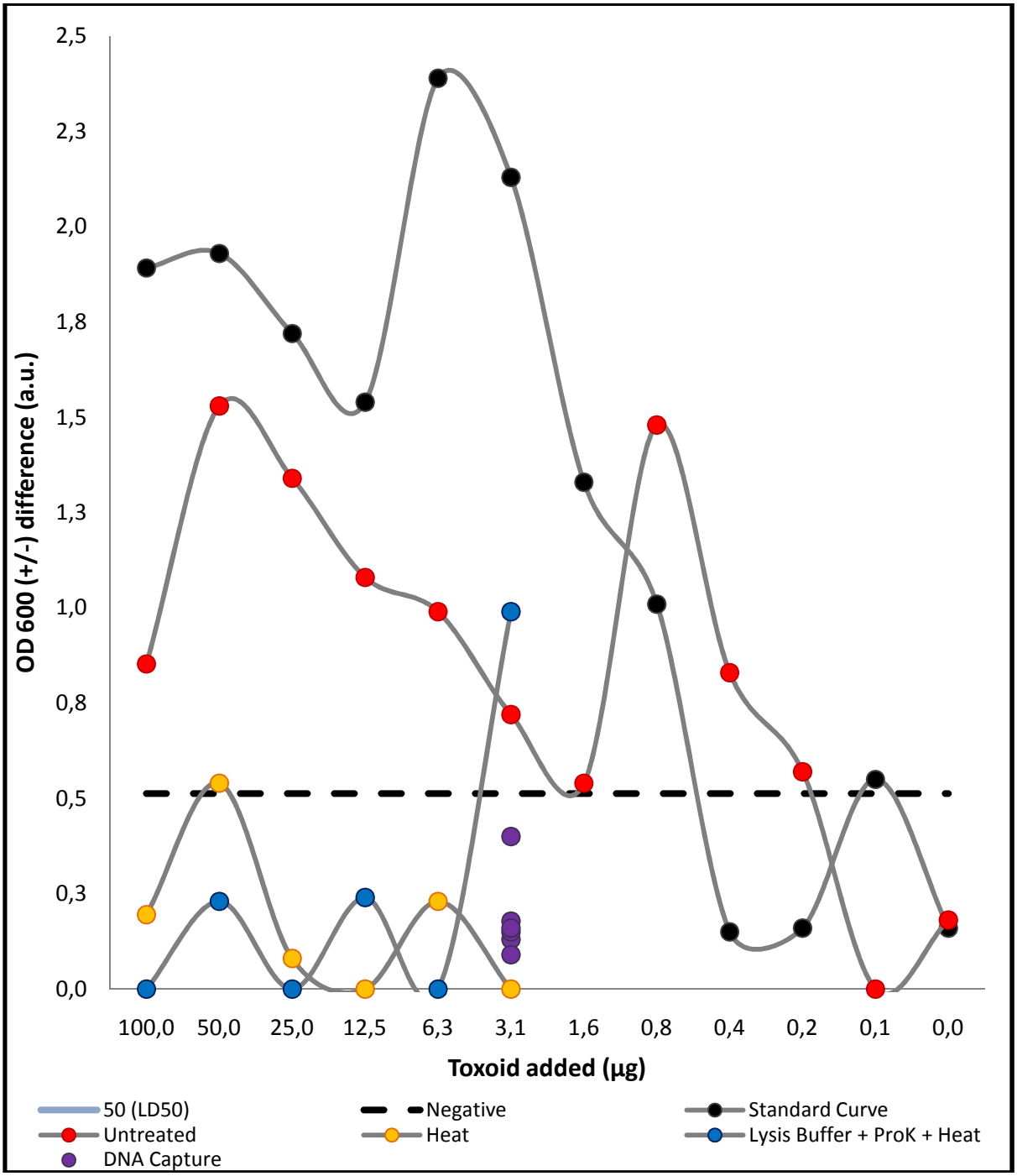

FIGURE 10 **Measured amount of recombinant ricin toxoid based on the standard curve and the absorbance obtained at 405 nm with the spectrophotometer determined by ELISA. Standard Curve = toxoid alone; Untreated = Toxoid mixed with blood without treatment; Heat = Toxoid mixed with blood incubated at 56 ºC alone; Lysis Buffer + proteinase K + Heat = toxoid mixed with blood incubated at 56 ºC with lysis buffer and proteinase K; DNA capture = extracted DNA with toxoid. $50LD_{50}$ was too high to illustrate compare to the rest of the data. (n=1).**

To summarize the findings, early steps in the standard DNA extraction methods eliminated infectious bacteria and degraded some toxins, but bacterial spores survived. Hence, a final filtration step is necessary to ensure removal of infectious material. These results are summarized in Table 8.

TABLE 8 Fate of spores, bacteria and toxins during the various steps of DNA isolation using the manual and automated extraction techniques.

	Bacillus anthracis	*Yersinia pseudotuberculosis*	Staphylococcal enterotoxin B	Botulinum toxin A	Ricin Toxoid
Positive growth control	+++	+++	+++	+++	+++
After heat incubation (56 °C)	+++	+	ND	(?)	X
Cell lysis	++	X	X	(?)	(?)
DNA capture	+				
After filtration	X				

Detection of biological agents was done by growth assay on TSA for bacterial spores and vegetative bacteria and by SDS-PAGE or ELISA for toxins. Experiments were repeated 3 times. X = biological agent has been degraded or eliminated; (?) uncertain; + = varying quantities from large amounts (+++) to small amounts (+) of the selected pathogen based on previous tables.

Manual versus automated extraction

The third objective of the thesis was to compare the quantity and quality of the DNA extracted using two distinct methods, either manual or automated DNA extraction. The efficiency of the techniques were first compared in the absence of contamination with selected biological agents, and then in the presence of the agents. The following experiments were also designed to determine the effects of these agents on DNA yields and human STR profile development. The earliest experiments, testing the use of the two lysis buffers to extract DNA from uncontaminated blood, suggested that the automated system was better by providing more DNA (Table 3B), and this has also been suggested by others (Bailey *et al.*, 2003). However, it has been shown that different extraction kits can influence the recovery rates of DNA (Hoile *et al.,* 2010). Furthermore, the persistence of materials that inhibit magnetic bead DNA extraction may require that samples be processed by phenol-chloroform DNA extraction which further decreases overall process efficiency (Barkham, 2006; Nagy *et al.,* 2005; Poon *et al.,* 2009). Additionally, when comparing methods, a fluctuation in DNA yields may be observed between donors, since the amounts of white blood cells in a control volume of blood may differ. Therefore, the standard deviation may vary between trials.

For all these reasons, the comparison of the manual and automated methods needed to be repeated. Blood from two donors was prepared to obtain duplicate samples from each of three volumes of blood ranging from 0.1 to 10 µL (total of 4 samples per volume). Buccal swab samples were taken from a single donor, but were divided into four aliquots. All sets of samples were set-up in parallel for extraction using the manual or automated systems. All experiments were repeated three times to give n = 12 for each volume of blood or saliva extracted by each method. In parallel, series of samples were also established containing 10^9 bacteria per sample, or 10 µL of a single dose of the selected toxin. Positive controls and negative controls as described previously were included. All samples were air-dried, then incubated in the lysis buffer with proteinase K overnight at 56 ºC in a water bath. The remainder of the DNA extraction was carried out as described previously for the manual and the automated methods. The final eluates containing DNA were quantified and analyzed at the RCMP forensic laboratory. The human DNA quantity was determined by calculating the average quantity of DNA (ng) isolated between donors in a final volume (100 µL) of elution

buffer. See Appendix A for quantification standard curves. Finally, STR analysis was carried out after half of the sample was tested for bacterial growth during the safety assessment.

The results of DNA extraction from uncontaminated samples in three experiments are shown in Table 9. It appeared that the automated extraction was more reliable and consistent, as larger amounts of DNA were obtained. The yields were more than tenfold-higher with the automated method as compared to manual extraction in the first three experiments. The differences in amounts of DNA in the first experiment were all statistically significant, while in the second experiment, the only significant difference was seen in saliva samples, and the third experiment, differences between methods were not significant. DNA extracted by the automated method had more success in profiling compared to manual extraction. A higher number of completed STR profiles across all three experiments, 73 out of 128 samples, was obtained for automated extractions compared to 23 completed profiles out of 128 samples for the manual extractions (Table 9). However, in the later experiments, presented in Table 10, it was observed that the amounts of DNA extracted and the completed STR profile quality were comparable between manual (103 out of 144 samples) and automated methods (84 out of 144 samples). In these experiments, amounts of DNA isolated were equivalent for the two methods between each of the volumes of blood, but were still lower in samples extracted by the manual method (average of ten-fold) for the unknown quantity of epithelial cells in the buccal swab, and these differences were significant for the fifth and the sixth experiments. Buccal swabs are inconsistent in the number of cells collected, compared to known amounts of blood, although as already noted, the number of cells in a given volume of blood can also vary considerably.

Based on these experiments, it can be concluded that the manual method was less consistent than the automated, but with practice, the efficacy of the manual method improved to a point where the DNA yields equalled those obtained using the automated protocol.

TABLE 9 **First evaluation of the manual extraction versus the automated extraction by comparing the quantity and quality of human DNA extracted from uncontaminated blood and buccal swab samples for three trials.**

	Manual extraction		Automated extraction	
Samples	Average DNA quantity (ng ± STDEV)	Completed STR profiles	Average DNA quantity (ng ± STDEV)	Completed STR profiles
First DNA extractions				
Blood (1 µL)	0.365 ± 0.252 0.844 ± 0.328	0/8 (4p)	1.726 ± 0.614 2.688 ± 1.060	7/8 (1p)
Blood (0.5 µL)	**0.073 ± 0.114** 1.317 ± 0.997	0/8 (2p)	**0.000 ± 0.000** 0.582 ± 0.093	4/8 (1p)
Blood (0.1 µL)	**0.092 ± 0.108** 1.503 ± 1.496	1/8 (2p)	0.633 ± 0.955 0.268 ± 0.192	6/8 (2p)
Buccal swab (unknown)	8.655 ± 6.456 11.46 ± 2.285	3/8 (2p)	44.95 ± 6.556 155.3 ± 52.58	4/8 (4p)
Second DNA extractions				
Blood (1 µL)	**0.191 ± 0.197** 0.415 ± 0.159 0.296 ± 0.360	0/12	5.089 ± 4.776 4.931 ± 1.626 3.236 ± 1.425	10/12 (1p)
Blood (0.5 µL)	**0.103 ± 0.063** **0.204 ± 0.183** 0.273 ± 0.140	0/12	1.521 ± 1.106 2.778 ± 1.556 1.451 ± 0.607	7/12 (1p)
Blood (0.1 µL)	**0.053 ± 0.039** **0.192 ± 0.098** **0.096 ± 0.111**	0/12	**0.149 ± 0.154** 0.385 ± 0.135 **0.246 ± 0.167**	0/12
Buccal swab (unknown)	2.394 ± 1.502 0.909 ± 0.506 1.681 ± 0.687	7/12	68.43 ± 39.06 71.50 ± 15.12 67.65 ± 23.28	12/12
Third DNA extractions				
Blood (1 µL)	91.03 ± 81.39 1.575 ± 0.829 25.25 ± 12.84	2/12 (1p)	9.575 ± 3.632 4.800 ± 0.616 8.050 ± 2.525	5/12 (2p)
Blood (0.5 µL)	13.17 ± 18.93 0.605 ± 0.420 5.050 ± 3.436	1/12 (1p)	4.625 ± 0.854 3.325 ± 1.132 0.940 ± 1.310	6/12 (2p)
Blood (0.1 µL)	10.52 ± 15.79 1.455 ± 2.430 2.175 ± 2.334	3/12 (1p)	1.675 ± 0.359 0.898 ± 0.482 0.345 ± 0.222	4/12 (1p)
Buccal swab (unknown)	83.75 ± 73.84 47.00 ± 33.06 616.8 ± 618.8	6/12 (1p)	732.5 ± 293.1 597.5 ± 288.1 1173 ± 130.5	8/12

DNA quantification are expressed as the average yield from two or three independent experiments, each done with duplicate samples from each of two donors (n=12). Bold red = DNA amount below the threshold of 0.250 ng. Numbers for DNA integrity represent the complete STR profiles of all extracted samples. (p) = Partial profile.

TABLE 10 **Second evaluation of the manual extraction versus the automated extraction by comparing the quantity and quality of human DNA extracted from uncontaminated blood and buccal swab samples for three trials.**

	Manual extraction		Automated extraction	
Samples	Average DNA quantity (ng ± STDEV)	Completed STR profiles	Average DNA quantity (ng ± STDEV)	Completed STR profiles
Fourth DNA extractions				
Blood (1 µL)	**0.211 ± 0.115** 1.150 ± 0.296 0.310 ± 0.188	8/12	1.643 ± 1.030 1.230 ± 0.350 1.950 ± 0.854	4/12 (2p)
Blood (0.5 µL)	0.270 ± 0.091 1.625 ± 0.350 **0.220 ± 0.146**	5/12	1.238 ± 1.328 0.540 ± 0.128 1.110 ± 0.229	8/12 (1p)
Blood (0.1 µL)	**0.150 ± 0.236** **0.144 ± 0.061** 0.348 ± 0.212	6/12 (1p)	**0.193 ± 0.030** **0.223 ± 0.086** **0.169 ± 0.110**	7/12 (2p)
Buccal swab (unknown)	2.675 ± 1.132 114.3 ± 39.31 19.00 ± 4.690	9/12	107.8 ± 75.94 88.50 ± 37.05 312.5 ± 55.00	12/12
Fifth DNA extractions				
Blood (1 µL)	0.556 ± 0.271 1.514 ± 2.132 2.320 ± 4.515	11/12	2.325 ± 0.597 2.423 ± 0.537 4.908 ± 1.435	11/12 (1p)
Blood (0.5 µL)	0.705 ± 1.014 0.361 ± 0.346 0.427 ± 0.316	8/12 (1p)	1.210 ± 0.196 0.956 ± 0.398 46.44 ± 89.045	5/12
Blood (0.1 µL)	1.433 ± 1.788 0.291 ± 0.249 **0.099 ± 0.117**	7/12 (2p)	**0.170 ± 0.087** 0.262 ± 0.219 0.625 ± 0.253	0/12 (1p)
Buccal swab (unknown)	10.54 ± 3.623 23.40 ± 9.156 11.96 ± 8.648	12/12	176.6 ± 71.85 323.3 ± 121.6 217.1 ± 147.1	12/12
Sixth DNA extractions				
Blood (1 µL)	1.180 ± 0.787 0.715 ± 0.452 4.820 ± 8.790	10/12 (2p)	1.675 ± 0.320 1.025 ± 0.481 1.303 ± 0.467	9/12 (2p)
Blood (0.5 µL)	0.895 ± 0.822 0.503 ± 0.401 0.275 ± 0.091	8/12	0.958 ± 0.169 0.983 ± 0.021 0.825 ± 0.304	3/12 (2p)
Blood (0.1 µL)	0.285 ± 0.054 0.383 ± 0.126 0.334 ± 0.166	7/12	0.313 ± 0.171 0.508 ± 0.470 0.425 ± 0.373	1/12 (2p)
Buccal swab (unknown)	166.0 ± 87.47 161.8 ± 69.42 139.5 ± 101.2	12/12	625.0 ± 114.7 337.5 ± 95.35 510.0 ± 115.8	12/12

DNA quantification are expressed as the average yield from three independent experiments, each done with duplicate samples from each of two donors (n=12). Bold red = DNA amount below the threshold of 0.250 ng. Numbers for DNA integrity represent the complete STR profiles of all extracted samples. (p) = Partial profile.

Effect of short-term exposure to bacteria and toxins on DNA extraction

As mentioned previously, the two extraction methods were compared to each other also using contaminated human samples. Blood and buccal samples were prepared as described above with the addition of *Pseudomonas aeruginosa*, *Yersinia pseudotuberculosis*, spores of *Bacillus anthracis*, or toxins, BoNT/A, SEB or ricin toxoid.

In these experiments, comparisons were made between the methods when samples were contaminated, and also between uncontaminated and contaminated samples. As with the uncontaminated samples, with each experiment completed, the differences in DNA yields and DNA integrity were less marked between the manual and the automated systems. In Table 11, representing the earliest experiment, the number of completed STR profiles with DNA isolated by the manual extraction (1 out of 48 samples) was lower than with DNA isolated using the automated version (11 out of 48 samples). The lack of STR profiles for the manually isolated DNA is likely related to the number of samples that did not meet the 0.250ng threshold, although DNA yields were not statistically different for the blood samples. Additionally, amounts of DNA from buccal swabs extracted by the manual and automated systems were statistically different in this experiment. In Table 12, the number of completed STR profiles were similar between the manual extraction (21 out of 48 samples) and the automated extraction (28 out of 48 samples), and all samples met the RCMP threshold for DNA yield. Statistical analysis revealed that differences in DNA yield obtained between extraction methods were significant for the 0.1 µL blood volume and the buccal swab samples. Similar results were observed in Table 13, Table 14, Table 15 and Table 16.

In the final experiment, the DNA isolated with the manual method was as good as DNA isolated by the automated method using both measures of quantity and quality (Table 17).

When DNA yields and DNA integrity were examined in contaminated samples compared to uncontaminated samples, the presence of *Pseudomonas aeruginosa* seemed to have a negative effect on DNA even with the automated system where yields were generally above the threshold (Table 11). When comparing the DNA yields from uncontaminated and

contaminated samples extracted by the automated method, two volumes of blood, 1 µL and 0.1 µL, and buccal swabs were statistically different, with reduced amounts of DNA in samples that had been contaminated with the bacteria. Furthermore, the numbers of completed profiles for uncontaminated samples (29 STR profiles) were higher than the contaminated samples (11 STR profiles). The decrease in the DNA yield could have been due to the fact that bacterial DNA preferentially bonded to the magnetic beads thus competing with human DNA, and therefore reducing the amounts of isolated human DNA.

When samples were contaminated with *Yersinia pseudotuberculosis*, there was a significant increase in the amounts of isolated DNA for all blood samples that were contaminated compared to uncontaminated samples. However, the number of completed STR profiles was similar between uncontaminated samples (23 completed STR profiles) and contaminated samples (28 completed STR profiles). Uncontaminated samples had more partial profiles (5 partial profiles) compared to the contaminated samples (2 partial profiles).

In summary, *Pseudomonas aeruginosa* had negative effects on the DNA quantity and quality, whilst *Yersinia pseudotuberculosis* had significant effects on DNA quantity in the smaller volumes of blood, but the DNA could be successfully interpreted via STR profiling. These results suggest that various vegetative bacteria have different effects on human DNA.

For blood and buccal swab samples contaminated with Bacillus anthracis spores, the results demonstrated very few effects of the presence of spores on the DNA yield and integrity (Tables 13 and 14). When comparing the DNA yields from uncontaminated versus contaminated samples extracted using the automated procedure, no significant differences were observed. The number of completed STR profiles for the contaminated samples were similar (70 out of 80 samples) compared to the profiles in the absence of *Bacillus* spores (68 out of 80 samples). Therefore, the short term presence of spores did not negatively affect the extraction of human DNA.

TABLE 11 **Quantity and quality of human DNA from uncontaminated samples and contaminated samples with *Pseudomonas aeruginosa* (10^9 bacteria/sample). The samples were subjected to either extraction method for three trials.**

	Manual extraction		Automated extraction	
Samples	Average DNA quantity (ng ± STDEV)	Completed STR profiles	Average DNA quantity (ng ± STDEV)	Completed STR profiles
Uncontaminated Samples				
Blood (1 µL)	**0.191 ± 0.197** 0.415 ± 0.159 0.296 ± 0.360	0/12	5.089 ± 4.776 4.931 ± 1.626 3.236 ± 1.425	10/12 (1p)
Blood (0.5 µL)	**0.103 ± 0.063** **0.204 ± 0.183** 0.273 ± 0.140	0/12	1.521 ± 1.106 2.778 ± 1.556 1.451 ± 0.607	7/12 (1p)
Blood (0.1 µL)	**0.053 ± 0.039** **0.192 ± 0.098** **0.096 ± 0.111**	0/12	**0.149 ± 0.154** 0.385 ± 0.135 **0.246 ± 0.167**	0/12
Buccal swab (unknown)	2.394 ± 1.502 0.909 ± 0.506 1.681 ± 0.687	7/12	68.43 ± 39.06 71.50 ± 15.12 67.65 ± 23.28	12/12
Contaminated Samples				
Blood (1 µL)	**0.056 ± 0.092** 0.313 ± 0.231 **0.165 ± 0.190**	0/12	1.521 ± 1.106 1.579 ± 0.703 **0.168 ± 0.155**	1/12
Blood (0.5 µL)	**0.219 ± 0.254** **0.103 ± 0.153** **0.058 ± 0.066**	0/12	1.263 ± 1.887 0.959 ± 0.205 0.367 ± 0.492	1/12
Blood (0.1 µL)	**0.011 ± 0.004** **0.104 ±0.084** **0.093 ± 0.168**	0/12	**0.079 ± 0.096** 0.312 ± 0.197 **0.127 ± 0.108**	0/12
Buccal swab (unknown)	0.579 ± 0.457 0.909 ± 0.506 0.537 ± 0.267	1/12	14.57 ± 17.20 28.14 ± 6.788 17.50 ± 12.73	9/12
Negative Controls				
Blank (no DNA)	**0.000 ± 0.000**	1/3 Contamination	**0.000 ± 0.000**	0/3

DNA quantification are expressed as the average yield from three independent experiments, each done with duplicate samples from each of two donors (n=12). Bold red = DNA amount below the threshold of 0.250 ng. Numbers for DNA integrity represent the complete STR profiles of all extracted samples. (p) = Partial profile.

TABLE 12 **Quantity and quality of human DNA from uncontaminated samples and contaminated samples with *Yersinia pseudotuberculosis* (10^9 bacteria/sample). The samples were subjected to either extraction method for three trials.**

	Manual extraction		Automated extraction	
Samples	Average DNA quantity (ng)	Completed STR profiles	Average DNA quantity (ng)	Completed STR profiles
Uncontaminated Samples				
Blood (1 µL)	91.03 ± 81.39 1.575 ± 0.829 25.25 ± 12.84	2/12 (1p)	9.575 ± 3.632 4.800 ± 0.616 8.050 ± 2.525	5/12 (2p)
Blood (0.5 µL)	13.17 ± 18.93 0.605 ± 0.420 5.050 ± 3.436	1/12 (1p)	4.625 ± 0.854 3.325 ± 1.132 0.940 ± 1.310	6/12 (2p)
Blood (0.1 µL)	10.52 ± 15.79 1.455 ± 2.430 2.175 ± 2.334	3/12 (1p)	1.675 ± 0.359 0.898 ± 0.482 0.345 ± 0.222	4/12 (1p)
Buccal swab (unknown)	83.75 ± 73.84 47.00 ± 33.06 616.8 ± 618.8	6/12 (1p)	732.5 ± 293.1 597.5 ± 288.1 1173 ± 130.5	8/12
Contaminated Samples				
Blood (1 µL)	187.4 ± 212.3 2.950 ± 2.596 2.325 ± 2.995	2/12	33.00 ± 2.708 33.25 ± 15.52 18.250 ± 2.062	10/12
Blood (0.5 µL)	27.85 ± 32.50 2.110 ± 3.407 2.144 ± 3.393	6/12	19,00 ± 4.320 10.38 ± 5.915 1.038 ± 0.838	8/12
Blood (0.1 µL)	1.815 ± 2.060 2.114 ± 3.209 2.299 ± 2.852	5/12 (2p)	5.100 ± 2.865 5.975 ± 5.456 6.725 ± 9.519	2/12 (1p)
Buccal swab (unknown)	117.0 ± 112.3 474.3 ± 483.0 289.0 ± 295.6	8/12	635.0 ± 103.4 1150 ± 493.3 877.5 ± 578.3	8/12 (1p)
Negative Controls				
Blank (no DNA)	**0.106 ± 0.122**	0/3	**0.082 ± 0.080**	0/3

DNA quantification are expressed as the average yield from three independent experiments, each done with duplicate samples from each of two donors (n=12). Bold red = DNA amount below the threshold of 0.250 ng. Numbers for DNA integrity represent the complete STR profiles of all extracted samples. (p) = Partial profile.

TABLE 13 **Quantity and quality of human DNA from uncontaminated samples and contaminated samples with *Bacillus anthracis* Sterne strain (10^6 spores/sample). The samples were subjected to either extraction method for three trials.**

	Manual extraction		Automated extraction	
Samples	Average DNA quantity (ng ± STDEV)	Completed STR profiles	Average DNA quantity (ng ± STDEV)	Completed STR profiles
Uncontaminated Samples				
Blood (1 µL)	0.365 ± 0.252 0.844 ± 0.328	0/8 (4p)	1.726 ± 0.614 2.688 ± 1.060	7/8 (1p)
Blood (0.5 µL)	0.073 ± 0.114 1.317 ± 0.997	0/8 (2p)	0.000 ± 0.000 0.582 ± 0.093	4/8 (1p)
Blood (0.1 µL)	0.092 ± 0.108 1.503 ± 1.496	1/8 (2p)	0.633 ± 0.955 0.268 ± 0.192	6/8 (2p)
Buccal swab (unknown)	8.655 ± 6.456 11.46 ± 2.285	3/8 (2p)	44.95 ± 6.556 155.3 ± 52.58	4/8 (4p)
Contaminated Samples				
Blood (1 µL)	0.088 ± 0.107 0.380 ± 0.111	2/8 (1p)	1.077 ± 0.477 2.376 ±1.057	7/8 (1p)
Blood (0.5 µL)	0.145 ± 0.098 0.616 ± 0.340	2/8	0.134 ± 0.268 0.484 ± 0.409	6/8
Blood (0.1 µL)	0.062 ± 0.086 0.745 ± 0.237	0/8 (1p)	0.065 ± 0.131 0.000 ± 0.000	4/8 (4p)
Buccal swab (unknown)	4.165 ± 1.358 8.046 ± 4.635	1/8 (1p)	54.44 ± 35.55 107.6 ± 25.67	6/8 (2p)
Negative Controls				
Blank (no DNA)	**0.000 ± 0.000**	0/2	**0.000 ± 0.000**	0/2

DNA quantification are expressed as the average yield from two independent experiments, each done with duplicate samples from each of two donors (n=8). Bold red = DNA amount below the threshold of 0.250 ng. Numbers for DNA integrity represent the complete STR profiles of all extracted samples. (p) = Partial profile.

TABLE 14 **Quantity and quality of human DNA from uncontaminated samples and contaminated samples with *Bacillus anthracis* virulent strain (10^9 spores/sample). The samples were subjected to either extraction method for three trials.**

	Manual extraction		Automated extraction	
Samples	Average DNA quantity (ng ± STDEV)	Completed STR profiles	Average DNA quantity (ng ± STDEV)	Completed STR profiles
Uncontaminated Samples				
Blood (1 µL)	1.638 ± 0.737 0.441 ± 0.511 27.76 ± 54.83	11/12 (1p)	5.265 ± 1.804 3.845 ± 2.403 4.813 ± 3.349	12/12
Blood (0.5 µL)	0.624 ± 0.273 **0.000 ± 0.000** **0.177 ± 0.160**	7/12 (5p)	1.204 ± 0.188 0.751 ± 0.272 1.190 ± 0.476	11/12 (1p)
Blood (0.1 µL)	0.422 ± 0.281 **0.174 ± 0.243** 42.89 ± 85.74	6/12 (5p)	0.459 ± 0.542 0.258 ± 0.313 0.436 ± 0.424	12/12
Buccal swab (unknown)	27.90 ± 13.36 8.824 ± 2.734 22.78 ± 28.47	12/12	273.9 ± 44.96 187.8 ± 63.11 213.4 ± 134.2	12/12
Contaminated Samples				
Blood (1 µL)	3.826 ± 3.188 **0.165 ± 0.238** 0.881 ± 0.594	11/12 (1p)	2.348 ± 0.320 5.311 ± 1.632 6.564 ± 2.943	12/12
Blood (0.5 µL)	2.730 ± 4.099 **0.108 ± 0.134** **0.170 ± 0.188**	8/12 (4p)	0.378 ± 0.165 **0.064 ± 0.228** 1.573 ± 0.807	12/12
Blood (0.1 µL)	0.982 ± 0.608 **0.000 ± 0.000** **0.208 ± 0.168**	1/12 (6p)	0.480 ± 0.347 0.487 ± 0.324 0.736 ± 0.360	11/12
Buccal swab (unknown)	18.36 ± 12.29 11.47 ± 5.727 20.94 ± 18.74	11/12 (1p)	176.1 ± 13.87 190.8 ± 38.12 220.6 ± 168.2	12/12
Negative Controls				
Blank (no DNA)	**0.240 ± 0.210**	0/3	**0.152 ± 0.122**	0/3

DNA quantification are expressed as the average yield from three independent experiments, each done with duplicate samples from each of two donors (n=12). Bold red = DNA amount below the threshold of 0.250 ng. Numbers for DNA integrity represent the complete STR profiles of all extracted samples. (p) = Partial profile.

The toxins BoNT/A, SEB and ricin toxoid also had no effects on DNA isolation from blood and buccal swab (Table 15, Table 16 and Table 17). Five contaminated blood samples, three for BoNT/A and two for SEB, were below the quantity threshold of 0.250 ng, but a Student T-test revealed no significant differences in the quantity of isolated DNA and the number of completed STR profiles when the results for all toxins were pooled: 84 out of 144 uncontaminated samples compared to 88 out of 144 contaminated samples.

The results of these experiments confirm the conclusion, that the automated system is no better for DNA isolation when compared to the manual, as long as the individual carrying out the latter is experienced. It was also concluded that the presence of *Pseudomonas aeruginosa* had some negative effect on the DNA yield or the development of full human STR profiles, and *Yersinia pestis* demonstrated an increased in DNA quantity. Some variations were noted in all samples contaminated with biological agents. However, other bacteria, bacterial spores or toxins that were tested did not compromise the DNA extraction procedures to an extent that affected DNA profiling.

TABLE 15 **Quantity and quality of human DNA from uncontaminated samples and contaminated samples with botulinum toxin A (1 µg/sample). The samples were subjected to either extraction method for three trials.**

	Manual extraction		Automated extraction	
Samples	Average DNA quantity (ng ± STDEV)	Completed STR profiles	Average DNA quantity (ng ± STDEV)	Completed STR profiles
Uncontaminated Samples				
Blood (1 µL)	**0.211 ± 0.115** 1.150 ± 0.296 0.310 ± 0.188	8/12	1.643 ± 1.030 1.230 ± 0.350 1.950 ± 0.854	4/12 (2p)
Blood (0.5 µL)	0.270 ± 0.091 1.625 ± 0.350 **0.220 ± 0.146**	5/12	1.238 ± 1.328 0.540 ± 0.128 1.110 ± 0.229	8/12 (1p)
Blood (0.1 µL)	**0.150 ± 0.236** **0.144 ± 0.061** 0.348 ± 0.212	6/12 (1p)	**0.193 ± 0.030** **0.223 ± 0.086** **0.169 ± 0.110**	7/12 (2p)
Buccal swab (unknown)	2.675 ± 1.132 114.3 ± 39.31 19.00 ± 4.690	9/12	107.8 ± 75.94 88.50 ± 37.05 312.5 ± 55.00	12/12
Contaminated Samples				
Blood (1 µL)	**0.223 ± 0.059** 1.983 ± 0.916 1.025 ± 0.577	6/12 (1p)	2.650 ± 0.777 1.985 ± 0.960 2.100 ± 0.483	8/12 (1p)
Blood (0.5 µL)	0.177 ± 0.243 1.200 ± 0.216 0.288 ± 0.145	9/12	1.190 ± 0.612 0.613 ± 0.038 1.450 ± 0.580	7/12 (1p)
Blood (0.1 µL)	0.638 ± 1.175 1.068 ± 0.944 **0.086 ± 0.073**	3/12 (1p)	**0.122 ± 0.080** **0.121 ± 0.097** **0.182 ± 0.132**	4/12 (1p)
Buccal swab (unknown)	7.550 ± 3.379 167.5 ± 38.62 29.40 ± 25.87	8/12 (2p)	217.5 ± 35.94 150.0 ± 31.62 100.8 ± 34.19	11/12 (1p)
Negative Controls				
Blank (no DNA)	**0.000 ± 0.000**	0/3	**0.000 ± 0.000**	0/3

DNA quantification are expressed as the average yield from three independent experiments, each done with duplicate samples from each of two donors (n=12). Bold red = DNA amount below the threshold of 0.250 ng. Numbers for DNA integrity represent the complete STR profiles of all extracted samples. (p) = Partial profile.

TABLE 16 **Quantity and quality of human DNA from uncontaminated samples and contaminated samples with staphylococcocal enterotoxin B (1 µg/sample). The samples were subjected to either extraction method for three trials.**

	Manual extraction		Automated extraction	
Samples	Average DNA quantity (ng ± STDEV)	Completed STR profiles	Average DNA quantity (ng ± STDEV)	Completed STR profiles
Uncontaminated Samples				
Blood (1 µL)	0.556 ± 0.271 1.514 ± 2.132 2.320 ± 4.515	11/12	2.325 ± 0.597 2.423 ± 0.537 4.908 ± 1.435	11/12 (1p)
Blood (0.5 µL)	0.705 ± 1.014 0.361 ± 0.346 0.427 ± 0.316	8/12 (1p)	1.210 ± 0.196 0.956 ± 0.398 46.44 ± 89.045	5/12
Blood (0.1 µL)	1.433 ± 1.788 0.291 ± 0.249 **0.099 ± 0.117**	7/12 (2p)	**0.170 ± 0.087** 0.262 ± 0.219 0.625 ± 0.253	0/12 (1p)
Buccal swab (unknown)	10.54 ± 3.623 23.40 ± 9.156 11.96 ± 8.648	12/12	176.6 ± 71.85 323.3 ± 121.6 217.1 ± 147.1	12/12
Contaminated Samples				
Blood (1 µL)	0.393 ± 0.260 0.396 ± 0.404 1.554 ± 2.418	10/12 (1p)	2.588 ± 0.312 2.458 ± 0.727 3.770 ± 2.106	11/12
Blood (0.5 µL)	**0.112 ± 0.093** 1.219 ± 2.101 0.499 ± 0.265	10/12	1.350 ± 0.185 0.905 ± 0.543 1.147 ± 0.152	5/12
Blood (0.1 µL)	**0.064 ± 0.099** 0.314 ± 0.135 0.453 ± 0.905	9/12 (1p)	**0.092 ± 0.054** **0.190 ± 0.072** 0.254 ± 0.133	3/12
Buccal swab (unknown)	5.153 ± 4.572 6.975 ± 4.141 8.930 ± 2.946	8/12 (2p)	167.6 ± 198.6 205.3 ± 107.0 137.4 ± 137.4	12/12
Negative Controls				
Blank (no DNA)	**0.067 ± 0.115**	0/3	**0.033 ± 0.029**	0/3

DNA quantification are expressed as the average yield from three independent experiments, each done with duplicate samples from each of two donors (n=12). Bold red = DNA amount below the threshold of 0.250 ng. Numbers for DNA integrity represent the complete STR profiles of all extracted samples. (p) = Partial profile.

TABLE 17 **Quantity and quality of human DNA from uncontaminated samples and contaminated samples with recombinant ricin toxoid (3 µg/sample). The samples were subjected to either extraction method for three trials.**

	Manual extraction		Automated extraction	
Samples	Average DNA quantity (ng ± STDEV)	Completed STR profiles	Average DNA quantity (ng ± STDEV)	Completed STR profiles
Uncontaminated Samples				
Blood (1 µL)	1.180 ± 0.787 0.715 ± 0.452 4.820 ± 8.790	10/12 (2p)	1.675 ±0.320 1.025 ±0.481 1.303 ± 0.467	9/12 (2p)
Blood (0.5 µL)	0.895 ± 0.822 0.503 ± 0.401 0.275 ± 0.091	8/12	0.958 ± 0.169 0.983 ± 0.021 0.825 ± 0.304	3/12 (2p)
Blood (0.1 µL)	0.285 ± 0.054 0.383 ± 0.126 0.334 ± 0.166	7/12	0.313 ± 0.171 0.508 ± 0.470 0.425 ± 0.373	1/12 (2p)
Buccal swab (unknown)	166.0 ± 87.47 161.8 ± 69.42 139.5 ± 101.2	12/12	625.0 ± 114.7 337.5 ± 95.35 510.0 ± 115.8	12/12
Contaminated Samples				
Blood (1 µL)	2.575 ± 1.457 0.708 ± 0.614 0.385 ± 0.119	10/12	1.775 ± 0.672 1.448 ± 0.425 1.680 ± 0.864	8/12
Blood (0.5 µL)	0.803 ± 0.594 0.480 ± 0.482 0.267 ± 0.140	5/12 (1p)	0.993 ± 0.671 0.970 ± 0.214 0.424 ± 0.406	4/12 (3p)
Blood (0.1 µL)	0.488 ± 0.438 **0.193 ± 0.015** **0.191 ± 0.154**	4/12 (2p)	0.538 ± 0.128 0.445 ± 0.265 0.283 ± 0.105	3/12
Buccal swab (unknown)	555.0 ± 326.6 119.8 ± 36.06 79.75 ± 49.94	12/12	592.5 ± 145.7 455.0 ± 86.60 575.0 ± 136.3	12/12
Negative Controls				
Blank (no DNA)	**0.071 ± 0.000**	0/3	**0.026 ± 0.023**	0/3

DNA quantification are expressed as the average yield from three independent experiments, each done with duplicate samples from each of two donors (n=12). Bold red = DNA amount below the threshold of 0.250 ng. Numbers for DNA integrity represent the complete STR profiles of all extracted samples. (p) = Partial profile.

Despite the conclusion, the use of an automated method is highly recommended during an investigation when one has unknown amounts of sample, or when staff may not be experienced with manual DNA extraction. Additionally, the automated system involves less handling of samples that may still contain spores. One possible concern with the automated system is contamination of the chamber during the extraction process. To confirm that the instrument and accessories used for the manual or the automated procedure did not spread the spores in the atmosphere, their exterior and interior were swabbed with a cotton swab dipped in TSB and plated on TSA (data not shown). The magnet block used for the manual extraction revealed the presence of spores. This could be related to the amounts of transfer done by the technician during the procedure. The exterior and interior of the automated extraction robot was free of spores, which is normal since the extraction steps are carried out inside a sealed tip. The used tips and the rest of the lysis solution were placed in TSB, and revealed the presence of some spores (5 of 6 samples).

Effect of long-term exposure to bacteria and toxins on DNA extraction

Although the robot was not consistently more efficient than the manual method, it was determined that automated DNA extraction provided more reliable results. Consequently, to complete the fourth objective, only the automated protocol was used. The final objective consisted of determining if prolonged exposure of biological samples to live bacteria, bacterial spores or toxins would affect the DNA yield. First, an experiment was completed to determine if an ambient environment could have an effect on DNA of non-contaminated blood and buccal swab samples incubated for one week. Table 18 shows that no statistical differences were observed in the DNA yields and DNA integrity when samples were held at room temperature for up to one week prior to extraction. At this point, non-contaminated samples should have been added to each experiment to compare the effects of prolonged exposure to contamination. However, only a positive control, referred as “aged blood”, was included in all subsequent experiments as a control for the effects of prolonged incubation of uncontaminated blood for seven days prior to DNA extraction.

TABLE 18 **Quantity and quality of human DNA extracted from samples incubated at room temperature.**

Samples	Average DNA quantity (ng ± STDEV)	Completed STR profiles
DAY 1		
Blood (1 µL)	33.58 ± 4.402 4.258 ± 2.030 4.185 ± 1.462	10/12 (2p)
Blood (0.5 µL)	11.36 ± 4.550 2.360 ± 0.820 1.960 ± 0.378	7/12 (3p)
Blood (0.1 µL)	21.94 ± 41.18 0.626 ± 0.133 1.118 ± 0.839	1/12
Buccal swab (unknown)	192.5 ± 68.19 262.3 ± 96.04 367.0 ± 65.96	11/12
DAY 3		
Blood (1 µL)	12.52 ± 2.543 20.38 ± 8.562 20.73 ± 6.443	12/12
Blood (0.5 µL)	8.050 ± 3.909 10.09 ± 4.012 8.560 ± 6.635	11/12
Blood (0.1 µL)	1.955 ± 1.023 2.462 ± 1.161 3.395 ± 3.665	7/12 (2p)
Buccal swab (unknown)	79.73 ± 21.67 498.8 ± 96.15 146.9 ± 99.63	9/12
DAY 7		
Blood (1 µL)	23.25 ± 4.535 20.68 ± 4.219 36.78 ± 9.807	12/12
Blood (0.5 µL)	9.758 ± 2.634 9.563 ± 4.809 12.72 ± 4.692	11/12
Blood (0.1 µL)	2.343 ± 0.493 3.355 ± 2.592 8.140 ± 10.91	8/12
Buccal swab (unknown)	100.0 ± 57.77 239.0 ± 51.75 189.4 ± 143.5	9/12 (2p)
CONTROLS (7 days exposure)		
Biological agents alone (10^9)	N/A	N/A
Aged blood (1 µL)	10.93 ± 2.200	3/3
Negative (Blank)	**0.000 ± 0.000**	0/3

DNA quantification are expressed as the average yield from three independent experiments, each done with duplicate samples from each of two donors (n=12). Bold red = DNA amount below the threshold of 0.250 ng. Numbers for DNA integrity represent the complete STR profiles of all extracted samples. (p) = Partial profile.

To determine the effect of longer periods of exposure to bacteria, spores or toxins on DNA in samples, blood from two donors and buccal swabs from one donor were seeded with 10^9 bacteria in 100 µL, or 10 µL of a selected dose of toxins. Analysis was done for samples contaminated with *Yersinia pseudotuberculosis*, *Bacillus anthracis* spores, BoNT/A, SEB and ricin toxoid. Contaminated samples and controls were placed in sterile tubes and air-dried, then incubated at room temperature for one, three or seven days before DNA was extracted and analyzed for quantity and quality. Amounts of DNA and development of full STR profiles were compared across days of exposure for each agent. To determine if bacteria or toxins were still present in the blood after seven days at room temperature, an experiment was performed (data not shown). The presence of live bacteria and spores was demonstrated by plating samples that had been incubated for seven days on TSA. Toxin was not found using the detection methods described previously. See Appendix B for quantification standard curves. See Appendix C for example of completed STR profiles.

There was a two-fold reduction in the DNA yield from the 1µL volume of blood contaminated with *Yersinia pseudotuberculosis* at day one relative to the "aged blood" control (Table 19). Further reductions in DNA yield, which were statistically significant, were observed in the 1 µL and 0.5 µL volumes of blood between day one and day three, and between all samples of day one and day seven. For the contaminated buccal swab samples, the DNA yields between day three and day seven were significantly reduced. Nevertheless, the amounts of DNA harvested from all samples were sufficient to proceed with DNA analysis, and the presence of *Yersinia pseudotuberculosis* for up to seven days did not affect the quality of DNA isolates as compared to DNA isolation at day one. Analysis revealed that the completed STR profiles were lower for day one (19 out of 48 profiles) compared to day three (31 out of 48 samples) and day seven (33 out of 48 samples). DNA isolated at day one resulted in more partial STR profiles. With these results included, the total interpretable profiles at day one were 37, not significantly different than at other times.

TABLE 19 **Quantity and quality of human DNA from blood and buccal swab samples exposed to *Yersinia pseudotuberculosis* for variable times.**

Samples	Average DNA quantity (ng ± STDEV)	Completed STR profiles
DAY 1		
Blood (1 µL)	38.75 ± 5.679 42.25 ± 5.560 24.50 ± 6.758	4/12 (7p)
Blood (0.5 µL)	25.25 ± 2.217 25.25 ± 5.315 12.75 ± 2.872	2/12 (8p)
Blood (0.1 µL)	4.275 ± 1.688 5.200 ± 0.469 2.850 ± 0.777	2/12 (2p)
Buccal swab (unknown)	990.0 ± 290.5 825.0 ± 229.0 440.0 ± 74.83	11/12 (1p)
DAY 3		
Blood (1 µL)	8.650 ± 2.301 11.68 ± 6.764 10.025 ± 4.964	11/12
Blood (0.5 µL)	5.475 ± 3.266 4.225 ± 2.304 5.500 ± 2.317	5/12 (1p)
Blood (0.1 µL)	1.140 ± 0.346 1.095 ± 0.381 1.750 ± 0.493	4/12 (1p)
Buccal swab (unknown)	365.0 ± 151.8 327.5 ± 61.31 363.5 ± 129.2	11/12
DAY 7		
Blood (1 µL)	18.50 ± 5.802 14.45 ± 17.44 4.515 ± 2.784	11/12
Blood (0.5 µL)	7.200 ± 4.569 4.450 ± 2.496 4.225 ± 1.926	7/12 (2p)
Blood (0.1 µL)	1.388 ± 0.581 1.225 ± 0.310 1.308 ± 0.509	3/12
Buccal swab (unknown)	132.5 ± 23.63 175.0 ± 43.59 146.0 ± 64.48	12/12
CONTROLS (7 days exposure)		
Biological agents alone (10^9)	3.011 ± 2.767	0/3
Aged blood (1 µL)	77.00 ± 55.22	3/3
Negative (Blank)	0.783 ± 1.068	0/3

Samples were seeded with 10^9 bacteria. DNA quantification are expressed as the average yield from three independent experiments, each done with duplicate samples from each of two donors (n=12). Bold red = DNA amount below the threshold of 0.250 ng. Numbers for DNA integrity represent the complete STR profiles of all extracted samples. (p) = Partial profile.

The presence of *Bacillus* spores, resulted in no reduction of amounts of DNA compared to the uncontaminated "aged blood" control samples (Table 20). Compared to DNA isolated from samples contaminated with other microbes, a higher number of samples fell below the recommended threshold of DNA yield to proceed with DNA analysis. But the yields, even in the control samples, were much reduced compared to other experiments, so perhaps it is not the spores but just the normal variation from experiment to experiment. Longer incubation periods with spores had no further effect on DNA quantity, but the number of completed profiles (out of 48 profiles) declined over the one week period, from 22 at day one, 21 at day three, and 12 at day seven.

The amounts of DNA isolated from the buccal swabs contaminated with BoNT/A were significantly lower at day three and day seven compared to day one of incubation (Table 21), while for the ricin toxoid, there was no significant difference between the samples (Table 22). Overall, the presence of BoNT/A or ricin toxoid in samples for increasing time resulted in only small changes in the amounts of DNA isolated or in the number of completed profiles.

For SEB, there was no decline in the DNA yields over time, but there were variations between samples (Table 23). Also, the seven days of exposure resulted in some degradation of DNA compared to one day exposure, as measured by the number of completed STR profiles. After seven days, the number of completed STR profiles was reduced for all blood samples and buccal swabs, as compared to samples incubated for the first and third day.

In summary, exposure to some biological agents reduced the amount of DNA after only three days of incubation. These factors are of the utmost importance when dealing with a crime scene involving biological weapons. Therefore, the extraction of contaminated human biological samples should be done prior to three days of exposure if possible to reduce the loss of DNA, therefore, decreasing the chance of DNA degradation.

TABLE 20 **Quantity and quality of human DNA from blood and buccal swab samples exposed to spores of *Bacillus anthracis* virulent strain for variable times.**

Samples	Average DNA quantity (ng ± STDEV)	Completed STR profiles
DAY 1		
Blood (1 µL)	0.431 ± 0.530 2.988 ± 3.531 0.312 ± 0.210	10/12
Blood (0.5 µL)	**0.115 ± 0.139** 1.023 ± 1.810 **0.083 ± 0.096**	2/12
Blood (0.1 µL)	**0.000 ± 0.000** **0.000 ± 0.000** **0.094 ± 0.188**	0/12
Buccal swab (unknown)	41.42 ± 30.26 67.76 ± 56.19 17.75 ± 35.50	10/12
DAY 3		
Blood (1 µL)	0.611 ± 0.499 0.273 ± 0.234 0.263 ± 0.416	10/12
Blood (0.5 µL)	0.306 ± 0.357 **0.087 ± 0.102** **0.158 ± 0.315**	3/12
Blood (0.1 µL)	0.269 ± 0.407 **0.071 ± 0.085** **0.000 ± 0.000**	0/12
Buccal swab (unknown)	15.91 ± 18.17 18.73 ± 14.50 75.76 ± 39.02	8/12
DAY 7		
Blood (1 µL)	1.754 ± 1.538 7.773 ± 9.536 2.820 ± 2.956	12/12
Blood (0.5 µL)	0.269 ± 0.432 1.149 ± 1.466 0.676 ± 1.353	0/12
Blood (0.1 µL)	**0.140 ± 0.280** **0.054 ± 0.107** 0.333 ± 0.385	0/12
Buccal swab (unknown)	15.06 ± 11.71 12.58 ± 15.33 12.75 ± 3.951	0/12
CONTROLS (7 days exposure)		
Biological agents alone (10^9)	**0.000 ± 0.000**	0/3
Aged blood (1 µL)	1.177 ± 1.038	3/3
Negative (Blank)	**0.000 ± 0.000**	0/3

Samples were seeded with 10^9 spores. DNA quantification are expressed as the average yield from three independent experiments, each done with duplicate samples from each of two donors (n=12). Bold red = DNA amount below the threshold of 0.250 ng. Numbers for DNA integrity represent the complete STR profiles of all extracted samples. (p) = Partial profile.

TABLE 21 **Quantity and quality of human DNA from blood and buccal swab samples exposed to botulinum toxin A for variable times.**

Samples	Average DNA quantity (ng ± STDEV)	Completed STR profiles
DAY 1		
Blood (1 µL)	16.50 ± 4.655 19.50 ± 10.47 13.75 ± 3.403	12/12
Blood (0.5 µL)	6.750 ± 2.897 8.275 ± 2.597 7.550 ± 1.377	12/12
Blood (0.1 µL)	2.125 ± 0.741 1.600 ± 0.424 1.550 ± 0.480	6/12 (1p)
Buccal swab (unknown)	487.5 ± 143.9 247.5 ± 9.574 312.5 ±110.9	11/12
DAY 3		
Blood (1 µL)	12.08 ± 2.239 17.25 ± 3.594 12.33 ±2.724	10/12 (2p)
Blood (0.5 µL)	8.100 ± 2.404 7.600 ± 1.476 5.650 ± 2.439	11/12 (1p)
Blood (0.1 µL)	1.625 ± 0.499 1.118 ± 0.335 1.068 ± 0.441	1/12 (3p)
Buccal swab (unknown)	207.5 ± 122.3 407.5 ± 68.50 196.8 ± 119.0	12/12
DAY 7		
Blood (1 µL)	11.80 ± 3.930 15.38 ± 5.121 20.78 ±15.39	12/12
Blood (0.5 µL)	9.075 ± 3.298 4.575 ± 1.706 6.825 ± 1.072	12/12
Blood (0.1 µL)	1.280 ± 0.431 1.575 ± 0.427 1.070 ± 0.286	0/12 (6p)
Buccal swab (unknown)	129.5 ± 95.64 170.3 ± 133.8 255.5 ± 154.2	12/12
CONTROLS (7 days exposure)		
Toxin alone (1 µL)	540.7 ± 917.5	0/3
Aged blood (1 µL)	7.967 ± 1.498	3/3
Negative (Blank)	0.395 ± 0.533	0/3

Samples were seeded with 1 µg. DNA quantification are expressed as the average yield from three independent experiments, each done with duplicate samples from each of two donors (n=12). Bold red = DNA amount below the threshold of 0.250 ng. Numbers for DNA integrity represent the complete STR profiles of all extracted samples. (p) = Partial profile.

TABLE 22 **Quantity and quality of human DNA from blood and buccal swab samples exposed to the recombinant ricin toxoid for variable times.**

Samples	Average DNA quantity (ng ± STDEV)	Completed STR profiles
DAY 1		
Blood (1 µL)	18.50 ± 9.469 10.78 ± 2.956 10.38 ± 4.507	12/12
Blood (0.5 µL)	5.650 ± 1.396 5.775 ± 1.075 3.900 ± 1.254	11/12 (1p)
Blood (0.1 µL)	0.678 ± 0.111 0.925 ± 0.492 0.790 ± 0.422	3/12 (2p)
Buccal swab (unknown)	175.0 ± 34.16 222.5 ± 100.8 152.3 ± 59.44	12/12
DAY 3		
Blood (1 µL)	11.95 ± 4.001 17.25 ± 8.461 14.33 ± 4.888	12/12
Blood (0.5 µL)	5.650 ± 1.646 11.53 ± 3.513 5.425 ± 2.877	11/12 (1p)
Blood (0.1 µL)	0.630 ± 0.314 0.988 ± 0.411 0.545 ± 0.197	2/12 (1p)
Buccal swab (unknown)	197.50 ± 60.21 125.0 ± 19.15 147.5 ± 28.72	11/12 (1p)
DAY 7		
Blood (1 µL)	15.25 ± 4.425 20.00 ± 2.309 15.95 ± 5.900	12/12
Blood (0.5 µL)	7.250 ± 1.949 6.850 ± 1.271 7.050 ± 1.794	12/12
Blood (0.1 µL)	0.768 ± 0.118 0.763 ± 0.299 0.571 ± 0.495	3/12
Buccal swab (unknown)	156.3 ± 55.58 117.5 ± 110.2 370.0 ± 224.7	11/12 (1p)
CONTROLS (7 days exposure)		
Toxoid alone (3 µg)	17.39 ± 29.12	0/3
Aged blood (1 µL)	10.03 ± 2.581	3/3
Negative (Blank)	**0.074 ± 0.127**	0/3

Samples were seeded with 3 µg. DNA quantification are expressed as the average yield from three independent experiments, each done with duplicate samples from each of two donors (n=12). Bold red = DNA amount below the threshold of 0.250 ng. Numbers for DNA integrity represent the complete STR profiles of all extracted samples. (p) = Partial profile.

TABLE 23 **Quantity and quality of human DNA samples exposed to staphylococcal enterotoxin B on blood and buccal swab samples for variable times.**

Samples	Average DNA quantity (ng ± STDEV)	Completed STR profiles
DAY 1		
Blood (1 µL)	32.83 ± 19.11 5.480 ± 1.111 3.673 ± 0.109	11/12
Blood (0.5 µL)	12.70 ± 5.121 4.715 ± 2.274 2.720 ± 1.721	8/12 (1p)
Blood (0.1 µL)	1.254 ± 1.058 1.463 ± 0.328 0.987 ± 0.710	0/12
Buccal swab (unknown)	126.1 ± 55.60 343.0 ± 69.59 659.8 ± 92.61	12/12
DAY 3		
Blood (1 µL)	16.55 ± 2.649 20.75 ± 5.368 10.51 ± 3.882	12/12
Blood (0.5 µL)	9.990 ± 1.077 9.063 ± 1.840 26.60 ± 42.75	12/12
Blood (0.1 µL)	0.841 ± 0.789 3.065 ± 0.779 2.433 ± 0.989	2/12 (4p)
Buccal swab (unknown)	71.73 ± 26.83 551.5 ± 67.58 85.48 ± 50.77	12/12
DAY 7		
Blood (1 µL)	15.72 ± 16.21 12.59 ± 8.387 7.847 ± 12.15	6/12
Blood (0.5 µL)	6.758 ± 7.743 7.625 ± 5.781 6.385 ± 7.455	5/12 (2p)
Blood (0.1 µL)	0.713 ± 1.425 3.210 ± 0.473 0.510 ± 0.866	0/12 (1p)
Buccal swab (unknown)	9.942 ± 19.57 301.5 ± 87.98 113.8 ± 224.1	6/12 (1p)
CONTROLS (7 days exposure)		
Toxin alone (1 µg)	**0.000 ± 0.000**	0/3
Aged blood (1 µL)	6.372 ± 5.618	3/3
Negative (Blank)	**0.000 ± 0.000**	0/3

Samples were seeded with 1 µg. DNA quantification are expressed as the average yield from three independent experiments, each done with duplicate samples from each of two donors (n=12). Bold red = DNA amount below the threshold of 0.250 ng. Numbers for DNA integrity represent the complete STR profiles of all extracted samples. (p) = Partial profile.

CHAPTER IV

Discussion

The recent upsurges in terrorist threats throughout the world have pressed governments, police and security agencies to prepare for any type of attack including the use of biological agents. Terrorists are becoming more sophisticated in their use of biological agents. Bioterrorists may select specific biological agents based on the capability to produce them on a large scale, ease of dispersement, and potential for widespread damage prior to detection (CDC, 2010). Indeed, spread of some biological agents could go undetected for weeks or months, as they are odourless and invisible when dispersed, making it increasing difficult to correlate cases of illness to the area of dissemination (Henderson, 2002).

As a result of the potential for possible attacks with biological weapons, security agencies must be prepared to collect and process evidence, such as biological samples (DNA) from sites contaminated with these agents, to identify the culprits (Hoile *et al.,* 2010; Wilkinson *et al.,* 2007). To obtain and safely transport evidence from a contaminated crime scene to a forensic laboratory, law enforcement agencies, such as the RCMP, require alternative field protocols for handling such specimens. The removal of infectious or toxic contamination would have to be conducted without compromising the integrity of human DNA.

The general goal of this work was to establish a SOP for the decontamination and extraction of human DNA contaminated with unknown biological agents recovered at a crime scene, in order for forensic institutes to safely analyze the collected evidence. The following objectives were established: 1) to confirm that the protocol previously developed for the removal of infectious bacteria and spores from human biological samples was able to eliminate or inactivate larger quantities of infectious material, 2) to determine if the DNA extraction procedure denatures toxins, 3) to compare the quantity and quality of extracted human DNA to determine which extraction protocol, manual or automated, would be preferable during the investigation of a crime scene contaminated with biological weapons, 4) and since biological weapons may be undetected for an undetermined period of time, the effects of immediate and prolonged exposure of biological samples to live bacteria, spores or toxins on quantity and quality of human DNA were examined.

Confirmation of loss or inactivation of pathogens/toxins

Since DNA extraction requires the presence of a lysis buffer and proteinase K to break the cell membranes, it was assumed that the heat treatment combined with the buffer and proteinase K would inactivate bacterial cells, but not spores. Spores can survive for decades in the ambient environment and are resistant to heat, microwaves, and ultraviolet light (Henderson *et al.*, 2002; Weinstein & Alibek, 2003). The original work to develop a protocol had shown that infectious material was already eliminated in samples seeded with *Staphylococcus aureus* prior to the filtration step, which suggested that bacteria were destroyed by the extraction process. Subsequently, it was shown that *Staphylococcus aureus* seeded into samples were being killed during a thirty minute incubation in lysis buffer (n = 40), but spores were not (n = 20) (Hause, 2007). In the original study, quantities of bacteria and spores were seeded into samples varying from 10^5 to 10^6 pathogens per sample. Therefore, the first objective of this project was to confirm that the original methodology of extracting DNA would remove larger numbers of contaminating bacteria and spores, and to establish the effects of each step of the DNA extraction process on infectivity.

It was found that an overnight incubation at 56 ºC in the presence of the lysis buffer with proteinase K did remove all infectivity from samples contaminated with the three tested bacteria, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Yersinia pseudotuberculosis* (n = 9). It was also shown that these bacteria were largely sensitive to the incubation at 56 ºC alone (8 out of 9 samples), although one sample contaminated with *Yersinia pseudotuberculosis* remained infectious due to a damaged filter.

Loss of infectivity of bacterial spores was also assessed at various stages of the extraction process using spores from two strains of *Bacillus anthracis*. Bacterial spores were seeded into samples at doses ranging from 10^6 (CL3 training) to 10^9 spores per sample. As expected, the incubation overnight at 56 ºC in the presence of the cell lysis buffer and proteinase K did not eliminate growth of *Bacillus anthracis* spores in any of the samples (n = 9), but the DNA extraction step did reduce the number of infectious spores by 71 % relative to the incubation at 56 ºC in PBS alone. However, the quantity of infectious spores was still too large to conclude that the samples were safe for manipulation without further

steps to remove the spores (Kendall *et al.*, 2008; Henderson *et al.*, 2002; Weinstein & Alibek, 2008).

These results are consistent with those of Hause (2007) who showed with *B. subtilis* spores that an incubation at 56 ºC in lysis buffer resulted in only a partial reduction of spore germination, while incubation at higher temperature (95 ºC) in the lysis buffer removed detectable infectivity in 39 out of 40 samples. However, proteinase K is inactivated by incubation at 70 ºC for fifteen minutes (Qiagen, 2009), so incubation at the higher temperature would need to be completed prior to the addition of the proteinase K, thus prolonging the extraction procedure. Additionally, due to the different types of samples to be analyzed the temperature in the original protocol was changed by the RCMP to 56°C to reduce the difficulty of working with sample, such as chewing gum and cigarette filters, at high temperatures. Therefore, the temperature in the methodology was kept at 56 ºC. Based on the resistance of spores to the conditions present during DNA extraction, the step of passing samples through the 0.22 µm filter was proposed and shown to be effective (Hause, 2007). In all of the experiments conducted with bacterial spores in this thesis (300 samples in total), filtration of the extracted DNA resulted in the absence of any infectious spores, as detected by absence of growth on TSA. Thus, the extraction and filtration steps are adequate and required to eliminate up to 10^9 bacteria or spores.

As previously mentioned, the number of bacteria and spores added to the samples were increased to 10^9 to reflect a possible production capacity of a bioterrorist. However, the number of pathogens found in a blood spot or saliva sample picked up at a crime scene could be as large as 10^{12} pathogens per sample. Therefore, a larger quantity of pathogens could interfere with the proposed methodology. The filter used could at one point be saturated and become clogged, which might result in lower DNA yields. The ruptured bacteria could prevent human DNA from passing through the filter pores or spores could go through a damage filter and contaminate the samples. Therefore, a growth assay should always be done after filtration to ensure that the unknown biological agent is indeed removed from human DNA sample before proceeding with the DNA analysis.

There are other bacterial agents with the potential to be used as biological weapons that have not been tested for removal in the protocol. However, it is expected that all bacteria and spores would be eliminated because they all possess similar features. The cell walls of bacteria are generally composed of a layer of peptidoglycan, phospholipids, lipopolysaccharides (Prescott *et al.,* 2003). In the RCMP extraction protocol, the incubation period should degrade bacterial cell wall membranes. The detergent present in the lysis buffer removes the lipids from the membrane, while the added proteinase K will degrade the proteins. Based on the demonstrated results, it is assumed that any types of microorganisms containing lipids and proteins, such as protozoa, fungi and protists, will be degraded during the incubation period (Weinstein & Alibek, 2003). The size range for protozoa is 1 to 300 µm, whilst for fungi it is 5 to 50 µm and for protist it is 2 to 20 µm (Prescott *et al.,* 2003). Therefore, the 0.22 µm filtration step added to the extractions process should remove these microorganisms if not previously destroyed by overnight incubation at 56 ºC with lysis buffer and proteinase K.

Since human DNA is able to pass through the 0.22 µm filter, it is only natural to assume that proteins will do the same. Therefore, the filter would not be able to physically remove the toxins selected for this project. However, the presence of detergent and proteinase K during an incubation period should inactivate the toxins (Dale & van Schantz, 2007). Using various techniques to detect the presence of toxins, it was shown that incubation in the lysis buffer with the presence of proteinase K was sufficient to reduce the amounts of SEB toxin to safe levels for manipulation and DNA analysis. However, for the other agents, the results were not as clear. For the ricin toxoid, the amount of toxoid detected was below 200 ng, well below a dose that would be toxic for the average adult, but both ELISA assays carried out were not as sensitive as reported by the manufacturer, and there was considerable variation in OD values between identical samples or within the standard curves. The lack of sensitivity in the ricin assay was likely related to the high background in the negative wells. This high background and fluctuation in the absorbance values could be related to the wash method, which was not as rigorous as might occur with an automated plate washer, and the incubation time period of washes. Indeed, like the manual DNA extraction, more experience may be required with the kits in order to obtain optimal results.

In addition to a lack of sensitivity, as determined from the standard curve, the ELISA assay for BoNT/A was not specific, and the reactivity observed was related to total protein. This reactivity did decline markedly after incubation in lysis buffer and proteinase K. However, because of the non-specific reactivity, it was not possible to rule out the presence of the toxin in the samples. One possibility for the high reactivity in these samples is that the ELISA was detecting fragments of the toxin even when partially degraded. If this was the case, Coomassie staining would not have been possible for the detection of BoNT/A, since the limit of detection was below the lethal dose of the toxin. Therefore, another detection technique should have been considered, such as silver staining, a LD_{50} assay with mice, a Bradford toxin assay or a Western blot.

As for bacteria, the amount tested of the toxin could have been larger to reflect more a concentration that could have been found in contaminated human samples in a situation of a bioterrorist attack. Though unlikely, the amounts of contaminating proteins could have saturated the quantity of proteinase K (1.2 mg/mL) in the lysis solution. A possible solution would be to increase the amount of proteinase K added, or other proteinase, when dealing with unknown biological samples to ensure total degradation of toxins.

Previous experimentation on contaminated blood with chemical weapons was initiated to observe the effect on DNA extraction. It was revealed that the RCMP DNA extraction process was also sufficient to neutralize the chemical weapons (Wilkinson *et al.,* 2007). Therefore, the first step of the standard DNA extraction protocol is efficient at removing chemicals and most biological contaminants from human biological samples, but a filtration step is necessary when handling samples contaminated with unknown agents to ensure removal of spores.

It is crucial to note that not all types of biological agents were tested. Previously, it was determined that the detergent and proteinase K present in the lysis buffer would degrade lipids and proteins, respectively (Dale & von Schantz, 2007). Therefore, the lipid toxins, such as *Mycobacterium ulcerans* toxin (George *et al.*, 1998), should also be degraded by the process to a point that is below the lethal dose. Along with other possible lipid or protein

toxins, there are also viruses that could be used for a bioterrorist attack. Since the size of viruses is 1/100th the size of bacteria, it is only natural to assume that the filter will not retain viruses (Prescott *et al.,* 2003). The content of viruses is usually protected by a capsid and/or an envelope (Prescott *et al.,* 2003). Many of the viruses with potential as biological weapons, such as Ebola virus, Lassa fever virus, Marburg virus, Rift Valley fever virus, West Nile virus and Yellow Fever virus, are enveloped viruses (Kendall *et al.*, 2008). Virus envelopes are mainly composed of lipids and proteins (Prescott *et al.,* 2003). Therefore, components in the RCMP lysis buffer, such as SDS, NaCl, Tris-HCl, EDTA, sarkosyl and DTT, and proteinase K, will degrade the lipid membrane, resulting in the loss of infectivity by preventing early steps of virus binding and entry into the host cells. Additionally, an increase in temperature or pH reduction of the buffer could automatically denature the viruses (De Filippi *et al.*, 1997). Thus, an additional step, such as the increase in temperature to 95 ºC for thirty minutes prior to the addition of proteinase K, could contribute to the inactivation of the viruses.

Viruses without an envelope possess a naked capsid that is mainly composed of proteins (Prescott *et al.,* 2003). Examples of non-enveloped viruses are hepatitis A virus, poliovirus, norovirus, rotavirus and human papillomavirus. Generally, non-enveloped viruses are more resistant to extreme conditions, such as exposure to solvents, detergents, acids and extreme temperatures (Roberts *et al.*, 1994). Therefore, a harsher technique could be used to denature or eliminate these viruses. An example of a method for inactivation of both non-enveloped and enveloped viruses is the use of a viricide. Certain biocides, such as alcohols, chlorine compounds, glutaraldehyde, peracetic acid, phenol ozone or metallic salts, which could be add to the proposed methodology for the removal of virus infectivity (Roberts *et al.*, 1994; Russell *et al.,* 2004). Extraction methods using phenol-chloroform-isoamyl alcohol mixture or guanidinium thiocyanate acid are known to denature viruses (Tsai *et al.*, 1995). There is also the use of a more physical approach to reduce viruses, such as a copper chelate affinity chromatography to physically remove them with ion-exchange chromatography for enveloped (reduction of 6.5 log) and for non-enveloped viruses (reduction of 4.0 log) (Roberts *et al.*, 1994).

Comparison of extraction methods

The literature and the removal of the selected biological agents from human biological samples allowed to proceed to the third objective: to investigate which of the two methods, manual or automated, would be preferable to extract human DNA *in situ*. Experiments were set-up so that the efficiency of the techniques was compared in the absence and presence of the selected biological agents, and to determine if the presence of contaminants would affect the efficiency of either method. It must be noted that when comparing DNA across samples within an experiment, a fluctuation in the DNA yield may be observed between the two donors, since the number of white blood cells may differ in a particular volume of blood. Also, there will be variation in the DNA isolated from samples collected from the same donor on different days. Therefore, the differences between methods were compared within the same experiment.

The early experiments with uncontaminated and contaminated samples, it appeared that the automated extraction performed better than the manual extraction, as determined by the significant difference in the average quantity of human DNA isolated as well as the completed STR profiles. It is known that different extraction techniques can influence the recovery rates of DNA (Hoile *et al.*, 2010; Zhou *et al.*, 1996). Factors that might contribute to the differences in the recovery rates are sample size, the concentration of the sample that a method can process, the formulation and/or the pH of the lysis buffers, the method of DNA isolation, as well as the interaction of DNA with particles, such as magnetic beads (Zhou *et al.*, 1996). The differences between manual and automated extraction described in this thesis could have been due to differences in the amounts of magnetic beads used in each kit; the automated system includes higher amounts of beads (Nagy *et al.*, 2005). However, it was observed in the later experiments that the amounts of DNA extracted and the profile quality were comparable between manual and automated methods. This suggested that in the hands of an experienced individual, the manual method could be as efficient as the robot. The same trend in improved performance was also observed with the robot. The number of successful profiles in the earliest experiments was not as good as in the subsequent experiments. Therefore, an experienced person in the forensic laboratory could obtain more accurate results.

The automated version may be more appropriate than the manual extraction if the work is proceeding on site, especially when working with dangerous biological agents (Bailey *et al.*, 2003), because it requires less handling of samples by workers, it reduces the amount of time of extraction and gives more consistent results from an inexperienced worker.

The presence of bacteria, spores or toxins in human blood or saliva had no effects on the capacity to extract DNA using either of the methods.

Effects of Exposure to Biological Agents on Human DNA

To determine if bacteria or toxins had an immediate effect on human DNA or interfered with the extraction or STR analysis, the quantity and results of analysis were compared between uncontaminated samples and samples seeded with bacteria *Pseudomonas aeruginosa*, *Yersinia pseudotuberculosis*, spores from two strains of *Bacillus anthracis*, and three toxins, which were dried and immediately extracted.

No differences had previously been observed in DNA extracted after immediate contamination with other vegetative bacteria, such as *Bacillus subtilis*, *Staphylococcus aureus*, *Yersinia pestis* and *Francisella tularensis* (Hause, 2007). Similarly, the presence of most of the agents used in experiments described in the present thesis had no significant effect on the yields of DNA or the DNA integrity, when present for short time periods. The smallest blood dilutions (0.1 µL) contaminated with all toxins were the only exception as they showed reduced integrity of isolated DNA. In this instance, because of the borderline amounts of DNA expected in such a low volume of blood, the reduced quantity and quality of human DNA could be associated with damage generated during the centrifugal filtration, as it was previously demonstrated that the filter did slightly lower the quantity (loss of 10 to 20 %), but not the quality (Hause, 2007).

Results from studies presented in the thesis did demonstrate that *Pseudomonas aeruginosa* had a more negative effect on the amounts of DNA isolated compared to other biological agents. In particular, there was a five-fold reduction in the amount of DNA

isolated from 1 µL blood, an average of two-fold reduction for the rest of the blood volumes and epithelial cells (buccal swab) seeded with this bacterium compared to uncontaminated samples, and the number of samples with successful STR analysis was also reduced. It was noted that in all three trials with this bacterium, the lysates obtained after the overnight incubation were extremely viscous unlike any of the other samples. The thick viscosity of the solution could have clogged the tips during extraction process. This phenomenon could have been generated from a combination of fragments and bacterial products, such as polysaccharides. Additionally, avirulent and virulent factors of the bacteria could have degraded the human DNA. The bacterial exoenzyme, ExoU, may have participated in the loss of DNA, since this toxin degrades the plasma membrane of eukaryotic cells, including white blood cells (Bender *et al.*, 2003; Prescott *et al.,* 2003).

There are other factors that could reduce the amount of isolated human DNA in extracted samples. When bacteria are present, perhaps there is saturation of the silica-coated magnetic beads with bacterial DNA instead of human DNA, but this should have no direct effects on DNA integrity, since the amplifications are specific to human genetic materials (Applied Biosystems, 2010; Frégeau *et al.,* 2010; Sensabaugh & Blake, 1993). However, low amounts of DNA could lead to allele dropouts resulting in the misinterpretations of DNA profiles, such as differences in homozygote and heterozygote alleles (Alonso *et al.*, 2004; Kline *et al.*, 2005). Variation in the profile integrity could also have been due to the presence of contaminants, such as small bacterial cell debris, that could have interfered with the amplification process. Finally, poorly executed extraction and fragment analysis techniques could have led to the low amounts of isolated DNA and the poorly obtained STR profiles. If bacterial controls with no blood revealed the presence of small quantities of DNA in the sample below the accepted DNA threshold for PCR ($\geq$ 0.250 ng), the quantities observed would represent background noise. Therefore, no contamination or cross-contamination was observed for these sets of samples. As well, primers used during quantification could have bonded non-specifically to bacterial DNA due to non-optimal annealing temperatures or primer dimerisation, thus providing misleading quantification result, which was previously seen with *Yersinia pestis* and *Francisella tularensis* (Applied Biosystems, 2010; Hause, 2007). A low annealing temperature would result in primers annealing a sequence other than

the true target, but it was not observed in any samples, whilst a high annealing temperature would yield low amounts of DNA. The former, which could have happened with *Pseudomonas aeruginosa* samples, can lead to nonspecific amplification and will consequently reduce the yield of the desired product.

Part of the fourth objective was to examine the effects of prolonged exposure of biological samples to live bacteria, spores or toxins on the quantity and quality of DNA obtained by extraction. Uncontaminated samples incubated at room temperature for up to one week prior to DNA analysis displayed no significant degradation in DNA compared to samples processed immediately. This was not unexpected, since the RCMP and police services isolate DNA from samples that are much older than one week on a routine basis. The original plan was to compare the trend observed within contaminated samples to these results. At this point, it was recognized that the experimental design was flawed. Uncontaminated samples should have been set-up and incubated in parallel to the contaminated samples to make the comparison more valid. Additionally, samples should have been created to compare the effects of possible degradation between day zero and day one. However, comparison between the three selected days permitted one to conclude some general observations with the help of the 1 μL uncontaminated blood control, labelled "aged blood".

For every biological agent, a total of 144 samples, were tested. Selected biological agents were able to survive in blood during the week incubation (data not shown). Obviously, spores would have survived for decades (Henderson *et al.*, 2002; Weinstein & Alibek, 2003). Nevertheless, none of the samples seeded with spores or bacteria contained infectious material after filtration following extraction. Samples contaminated with *Yersinia pseudotuberculosis* revealed some loss of DNA after the third day of exposure, compared to day one, and to the "aged blood" control, but not enough to prevent or affect STR analysis. For samples exposed to *Bacillus* spores, the amounts of DNA were often below the recommended threshold to proceed. However, because the amount of DNA isolated from the positive control of "aged blood" incubated without contamination was relatively the same as the contaminated samples, the presence of the bacterial spores might not have played a role

in the low amounts of human DNA isolated in these experiments. Evidently, bacterial spores could have adhered to the magnetic beads prior to human DNA. Therefore, the amounts of human DNA isolated would have been reduced. This could also explain the slight difference between the "aged blood" control and the contaminated samples.

The toxins used in these experiments were not anticipated to have direct effects on DNA, but they could have detrimental effects on the viability of cells within the human samples, which in turn would reduce DNA yields. The longer exposure to BoNT/A or ricin toxoid had no effect on the amounts of DNA isolated or in the number of completed STR profiles as compared to the "aged blood" controls. However, because the toxoid does not have the biological effect of ricin, which inhibits protein synthesis, there is still a possibility that the toxin could affect DNA yields and integrity if contaminating blood or epithelial cells from a buccal swab. Therefore, to further characterize the effects of ricin on human DNA, the authentic toxin should be used. For SEB, seven days of exposure resulted in some degradation of DNA compared to the first day. Interestingly, on the seventh day of exposure, the reduction in the number of completed STR profiles was significant. SEB is an enzyme secreted by *Staphylococcus aureus* during the invasion of the host to acquire nutrients for bacterial growth by lysing local host cells (Dinges *et al.,* 2000; Kendall *et al.,* 2008; Roberts *et al.,* 2000). The cytotoxic function of SEB could lead to organ failure, and some degradation in human DNA based on the above literature. As clearly demonstrated with the SEB toxin, DNA degradation is not necessarily associated with reduction in DNA quantity.

While these results show limited effects of prolonged exposure to bacteria, spores or toxins on the efficacy of human DNA extraction, longer incubation times in the ambient environment or in different conditions could have generated different results. Indeed, native DNA does degrade to smaller fragments. The process can be influenced with time by environmental factors, such as sunlight and temperature. An increase in temperature could spontaneously damage DNA by the dissociation of strands, whilst low temperatures would not affect DNA. Exposure to UV-light could generate base modification, such as pyrimidine dimers, and cross-linking between DNA strands to proteins, whilst aging by itself could cause base modification, strand scission and depurination (Kobilinsky, 1992; Sensabaugh &

Blake, 1993). Additionally, DNA degradation was readily apparent in the smaller volumes of blood, which might imply that this particular volume of blood (0.1 µL) is unreliable for detection of profiles, since the amounts of white blood cells vary significantly from sample to sample.

Future directions

Generally, it is accepted that automated extraction should be more efficient and consistent for the extraction of human DNA, whatever the conditions. The use of automation reduces error due to the reduction of human manipulation, while being able to extract a larger number of samples. Currently, the standard for DNA extraction at the RCMP and similar agencies is to use automation for various types of samples. However, some samples, for instance cigarettes butts and chewing gum, collected from crime scenes require additional preparation (removal of substrate) or extraction method (phenol-chloroform). The fact is that various buffers or technique applications might change the quantity of isolated DNA. Also, these types of changes might affect the number of completed STR profiles from the collected evidence. Therefore, various automated extractions should be tested to understand which of these techniques would be more efficient to incubate and extract samples, taken from a crime scene contaminated with biological weapons, to reduce the possible exposure to environmental bacteria.

During the validation of the developed methodology, distinct observations were made on the effects of different vegetative bacteria, bacterial spores and toxins on their inactivation or elimination. For this reason, it would be beneficial to test other species of biological agents. Also, filter saturation or clogging was not observed with the quantity used during the experiments. However, a higher concentration of pathogens could lead to a decontamination failure. Additionally, there are more than bacteria, bacterial spores, and toxin which could be used for biological weapons. Therefore, protozoa, fungi, protists and viruses should be taken into consideration to determine if the developed method would eliminate or inactivate these types of agents. Various effects on the human DNA were observed with different types of bacteria and toxins. The effect of prolonged exposure of human samples to pathogens was examined, but not with all types of microbial pathogens and toxins.

Incubation, extraction and analysis of the samples were done in a timely fashion. It would be interesting to investigate the effects of prolonged exposure time, storage and shipping conditions on various sample types, with the presence of contamination, to evaluate if it would be more beneficial to process the samples at the crime scene or in a contained laboratory.

Conclusions

The method originally proposed by Hause (2007) was validated. Indeed, the extraction method proposed is a good alternative to handle and extract samples contaminated with as many as 10^9 bacteria or spores, or a range of the median lethal dose of the toxins. Overnight heat treatment at 56 ºC in the presence of a lysis buffer and proteinase K is sufficient to inactivate or remove most of the selected pathogens. However, the added filtration step after the extraction of the contaminated samples is necessary to remove bacterial spores. The extraction process clearly demonstrated that a total of 1,400 samples contaminated with three different bacteria, two sets of spores, and some toxins were safe for further analysis. Assays with BoNT/A and ricin toxoid were inconclusive, but the literature supports that DNA extraction eliminates lipids and proteins.

The experiments demonstrated that an automated protocol would be preferable to isolate human DNA contaminated with biological weapons, because of its efficiency and consistency demonstrated throughout this project. Manual extraction is a good alternative to extract DNA, if one is proficient at it. Robotic extraction would also decrease contact with the contaminated samples, rendering the environment safer for the manipulator. Some reduction in the amounts of DNA was noted between uncontaminated and contaminated samples, which would indicate that the isolation of DNA using magnetic beads is not specific to human DNA or its association with the degradation of human DNA by pathogens. To confirm the safety of the samples, plating out onto TSA or a biological assay should be carried out to ensure that the unknown pathogens have been removed.

Finally, exposing human biological samples to the selected pathogens does increase the chance of degradation. Some pathogens, such as vegetative bacteria, had a greater effect on the isolated quantity of DNA, than the DNA integrity itself, whilst toxins had effects on human DNA degradation, but not on the quantity. The results of experiments examining the effects of bacterial spores on the quantity and quality of human DNA for prolonged exposure were inconclusive because of a poor extraction results from the control samples. In brief, after seven days of exposure to the pathogens, some negative effects were apparent. Therefore, to obtain optimal amounts of DNA as well as good STR profiles, it is recommended to collect and extract samples as soon as possible to reduce the chances of DNA degradation or the loss of genetic material, which would lead to a lack of amplification, and to the reduction of interpretable profiles.

The main hypothesis of this research was to investigate a standard protocol for extracting DNA which could possibly denature toxin, bacteria, and eliminate infectious bacterial spores from biological samples during extraction of human DNA. This process should be done without compromising the human DNA in the samples using adequate measures for the removal of infectious material, including bacterial spores. Based on the following model (Figure 11), a small robotic instrument would provide higher and more consistent yields of DNA in a field situation.

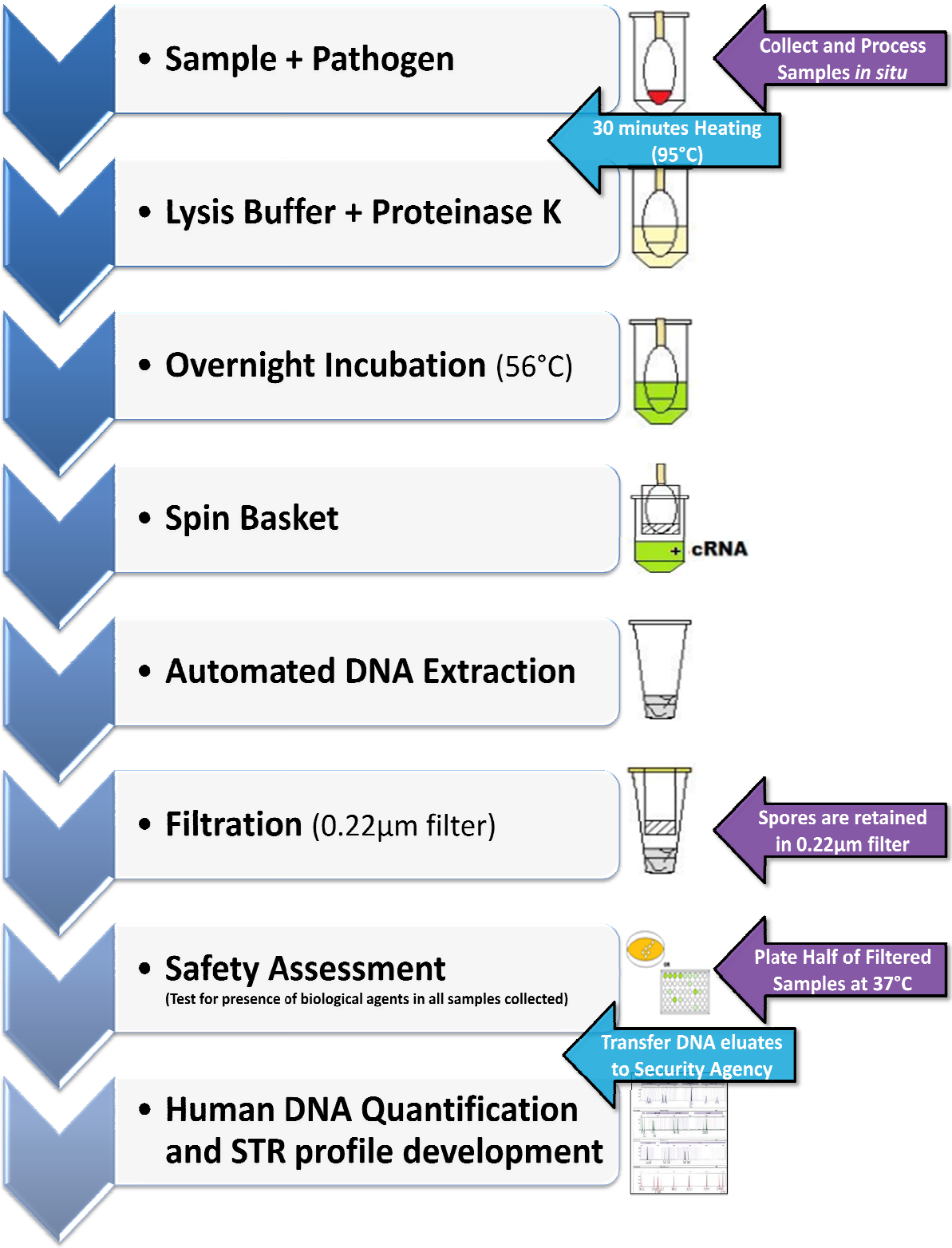

FIGURE 11 **Proposed human DNA extraction model for contaminated samples with biological weapons.**

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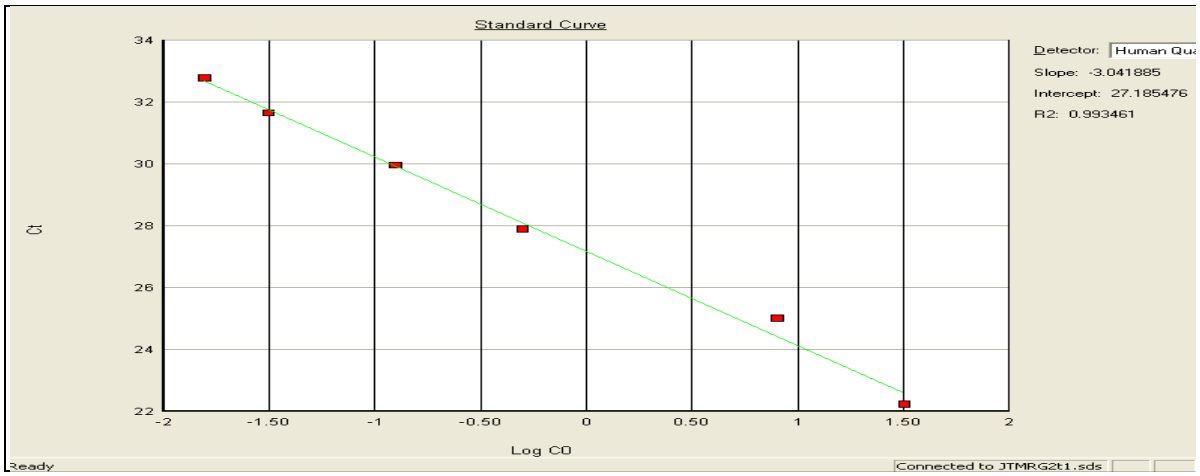
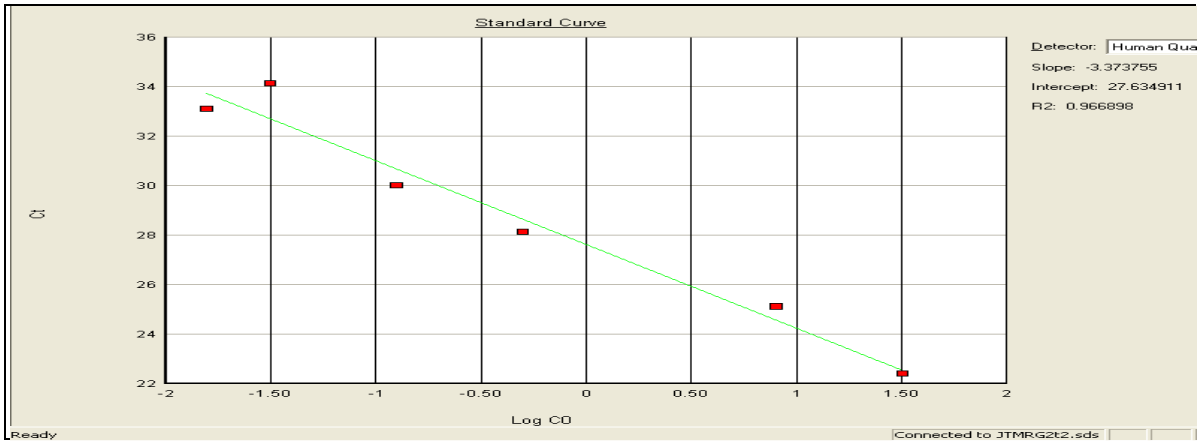
APPENDIX A

STANDARD CURVES for QUANTIFCATION of MANUAL vs. AUTOMATED SAMPLES

Trial 1



Trial 2



Trial 3

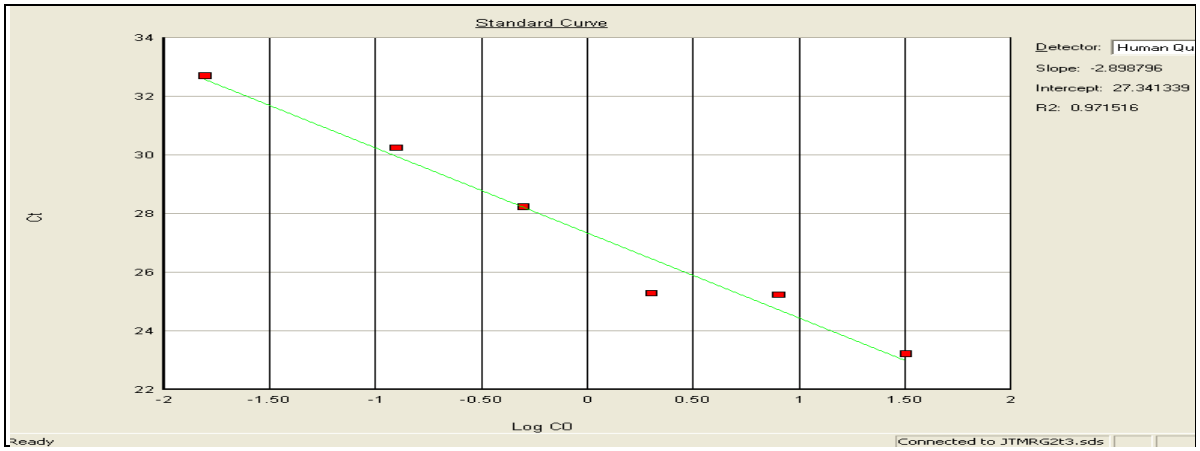
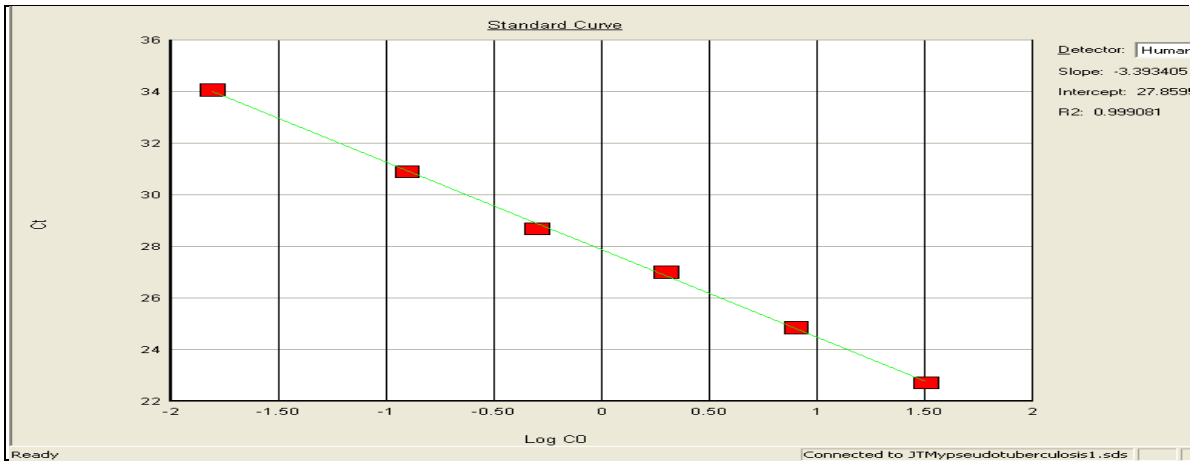
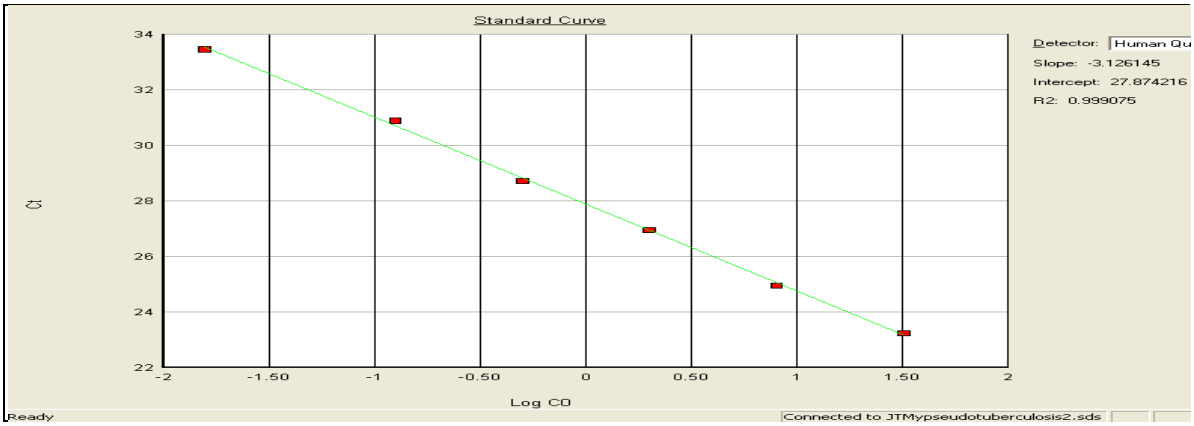


FIGURE A1 **Standard curve for DNA quantification of contaminated samples with *Pseudomonas aeruginosa*. C_T: threshold cycle of a specified amplification plot that occurs when the fluorescent signal increases beyond the value of the threshold setting.**

Trial 1



Trial 2



Trial 3

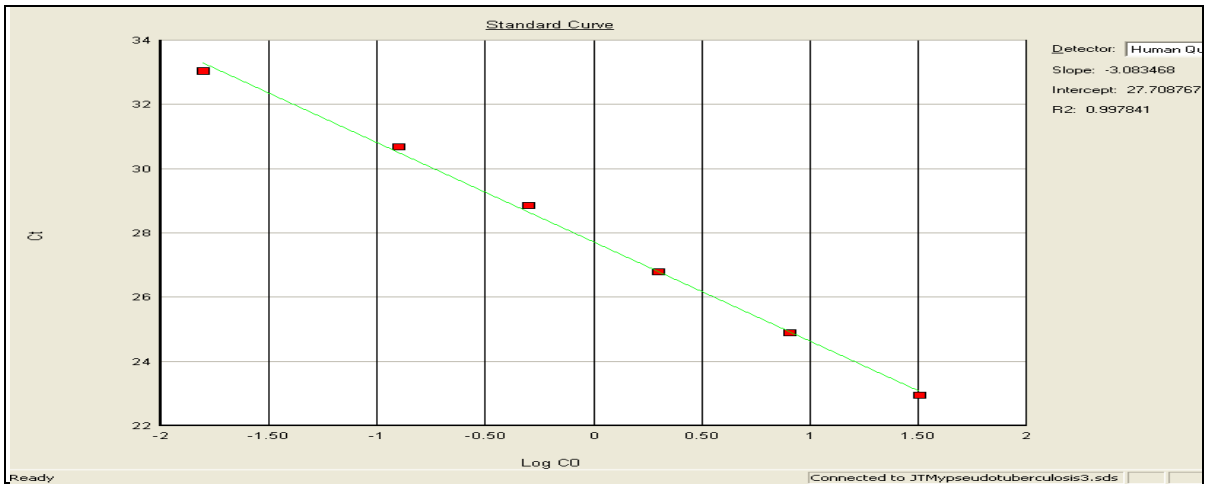
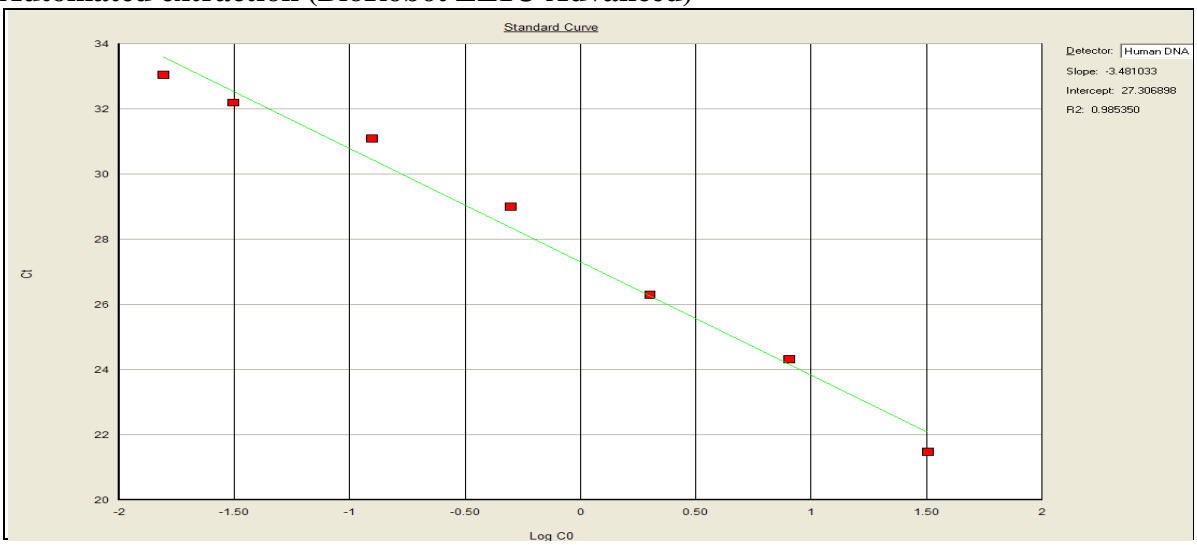


FIGURE A2 **Standard curve for DNA quantification of contaminated samples with *Yersinia pseudotuberculosis*. C_T: threshold cycle of a specified amplification plot that occurs when the fluorescent signal increases beyond the value of the threshold setting.**

Automated extraction (BioRobot EZ1® Advanced)



Manual extraction (DNA IQ System™)

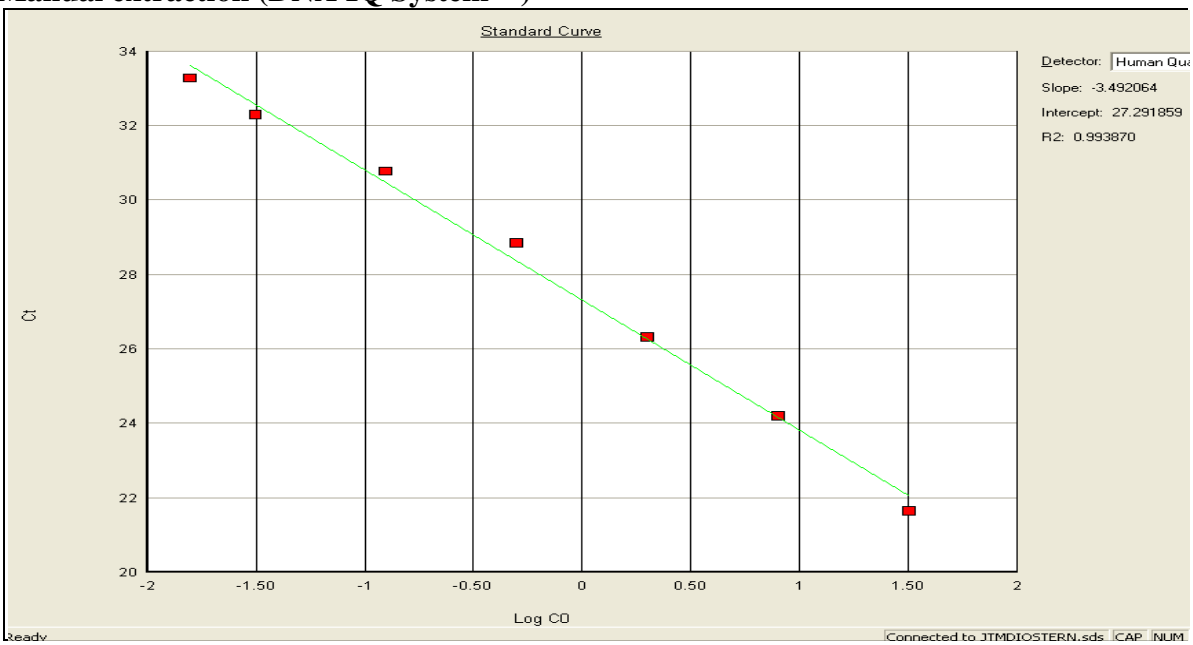
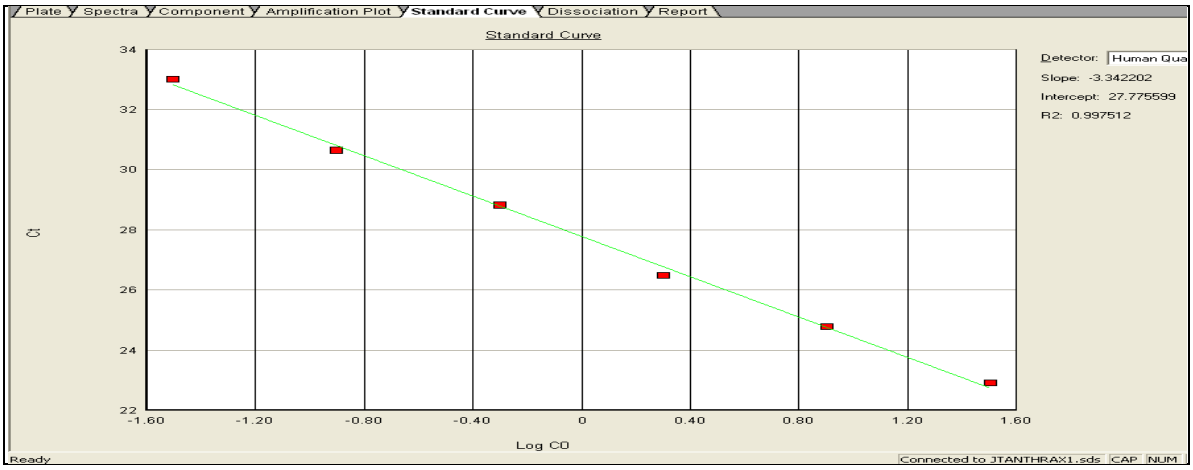
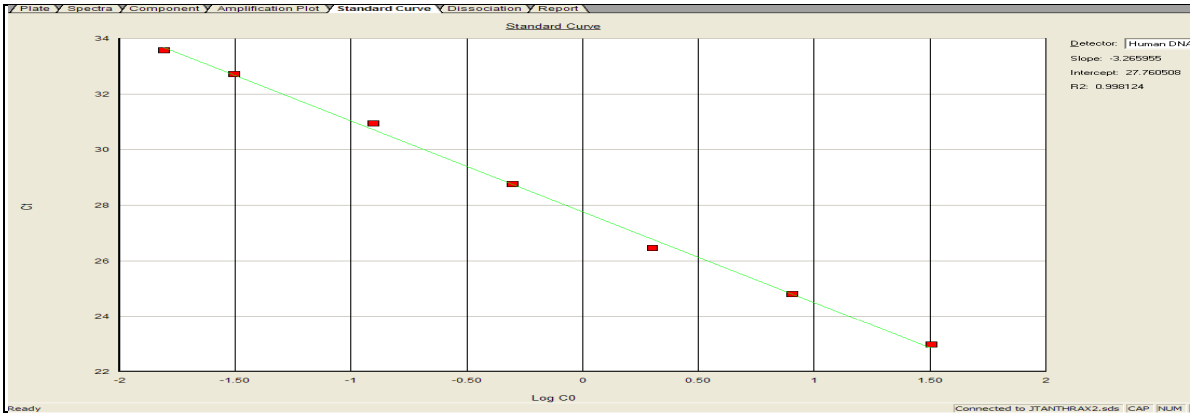


FIGURE A3 **Standard curve for DNA quantification of contaminated samples with spores of *Bacillus anthracis* sterne strain. C_T: threshold cycle of a specified amplification plot that occurs when the fluorescent signal increases beyond the value of the threshold setting.**

Trial 1



Trial 2



Trial 3

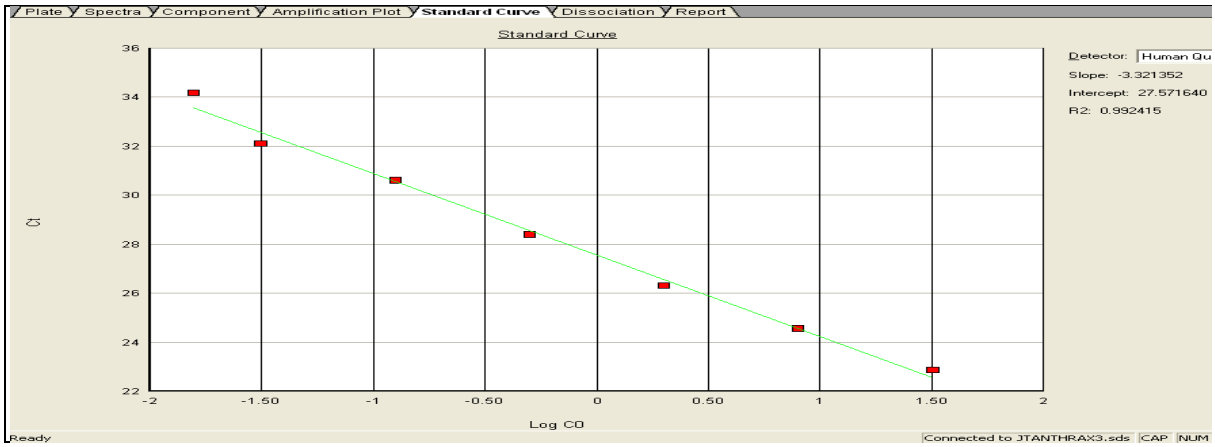
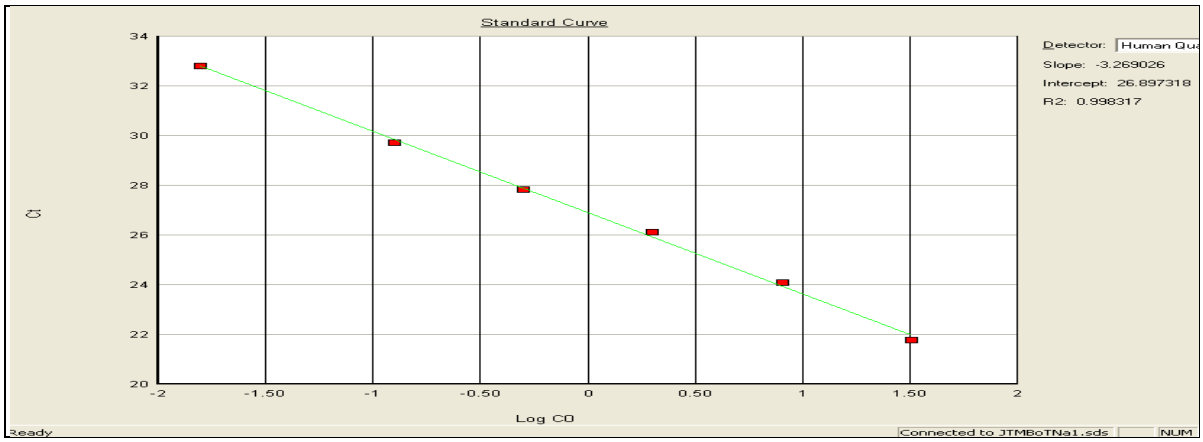
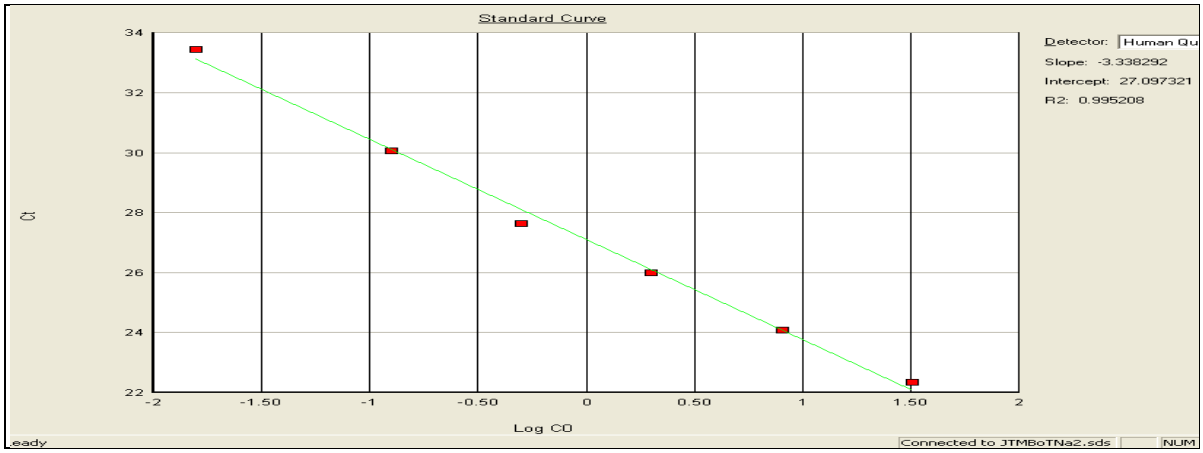


FIGURE A4 **Standard curve for DNA quantification of contaminated samples with spores of *Bacillus anthracis* virulant strain. C_T: threshold cycle of a specified amplification plot that occurs when the fluorescent signal increases beyond the value of the threshold setting.**

Trial 1



Trial 2



Trial 3

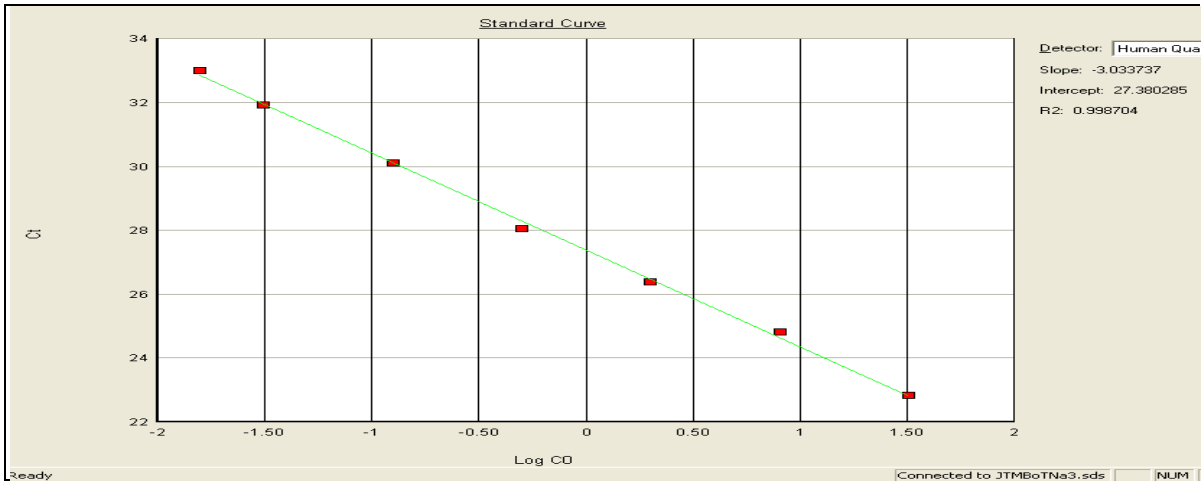
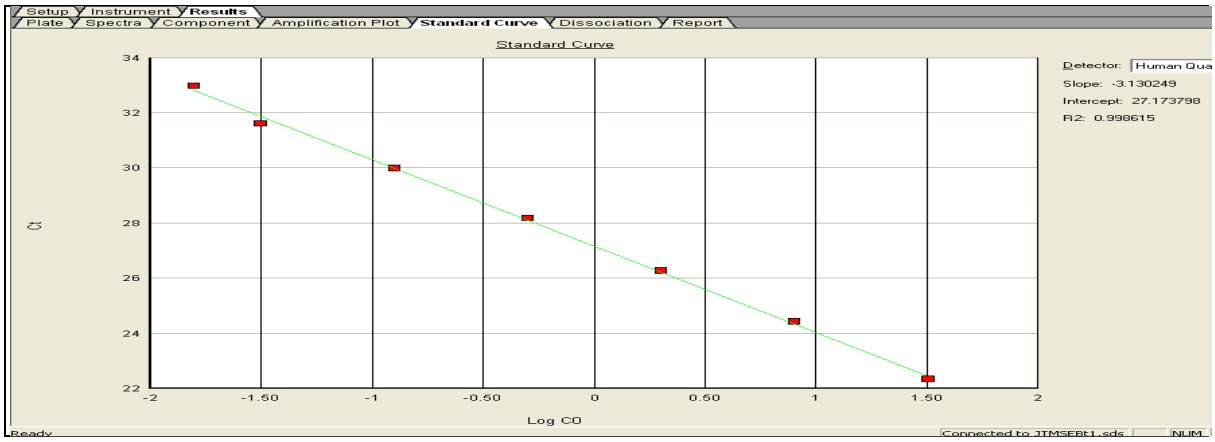
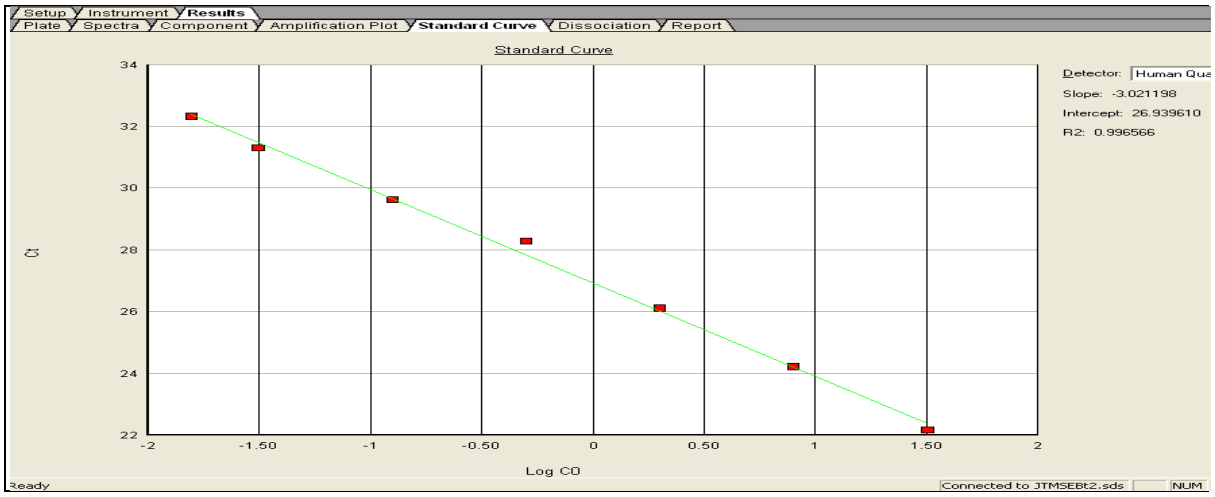


FIGURE A5 **Standard curve for DNA quantification of contaminated samples with botulinum toxin A. C_T: threshold cycle of a specified amplification plot that occurs when the fluorescent signal increases beyond the value of the threshold setting.**

Trial 1



Trial 2



Trial 3

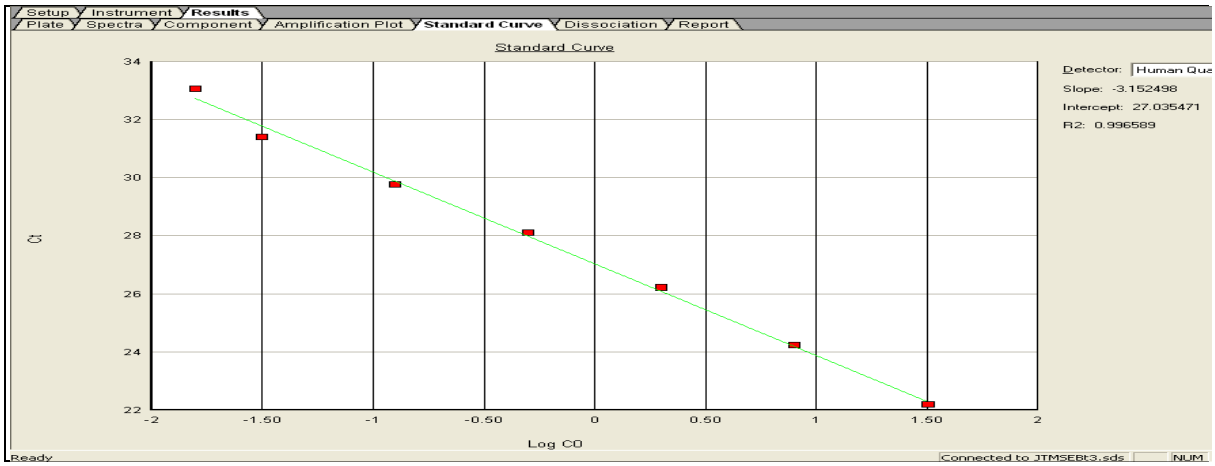
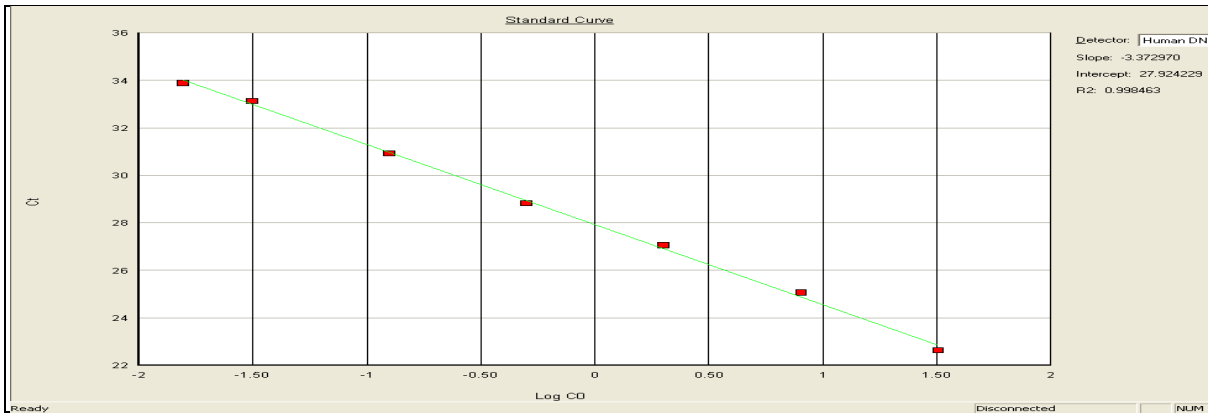
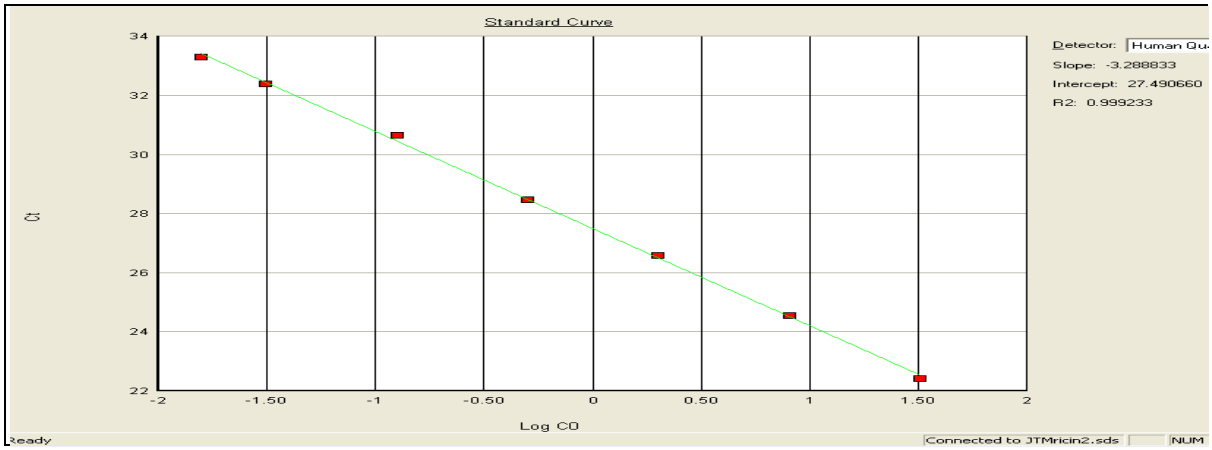


FIGURE A6 **Standard curve for DNA quantification of contaminated samples with Staphylococcocal enterotoxin B. C_T: threshold cycle of a specified amplification plot that occurs when the fluorescent signal increases beyond the value of the threshold setting.**

Trial 1



Trial 2



Trial 3

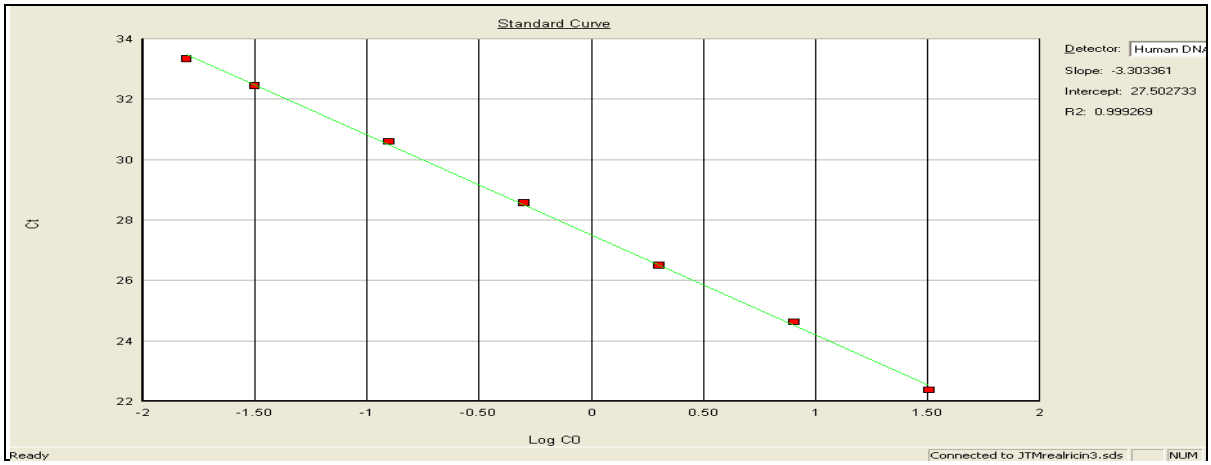
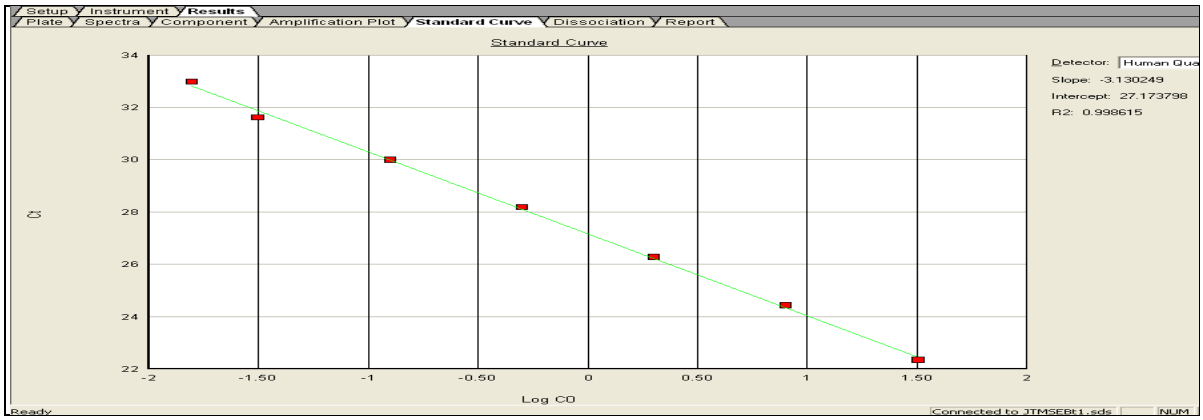


FIGURE A7 **Standard curve for DNA quantification of contaminated samples with the recombinant ricin toxoid. C_T: threshold cycle of a specified amplification plot that occurs when the fluorescent signal increases beyond the value of the threshold setting.**

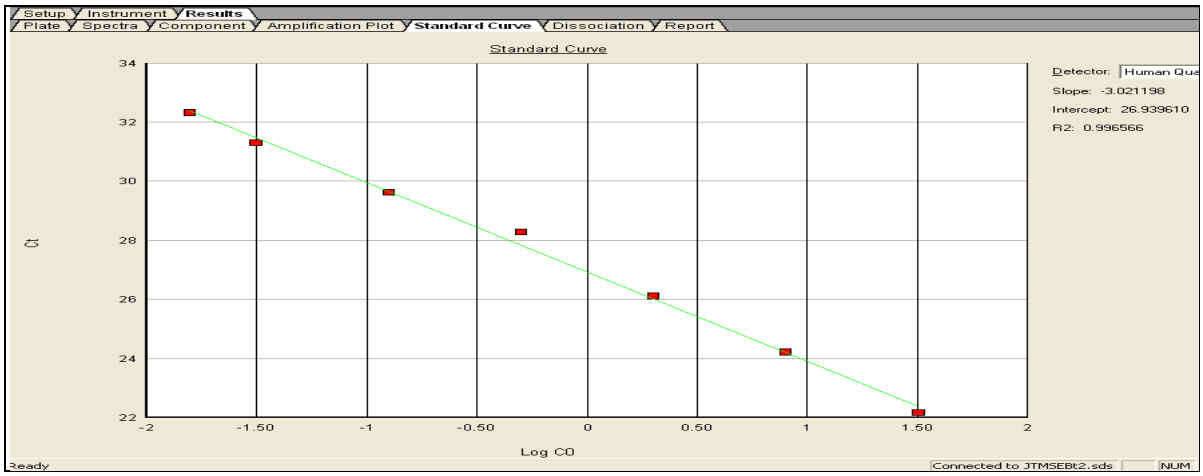
APPENDIX B

STANDARD CURVES for LONG TERM EXPOSURE to BACTERIA and TOXINS on DNA EXTRACTION

Trial 1



Trial 2



Trial 3

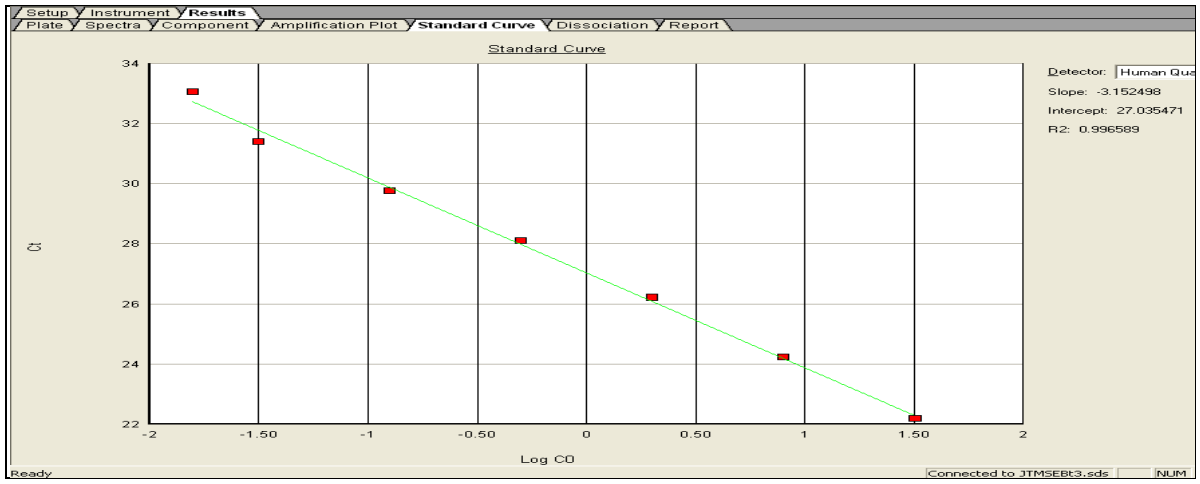
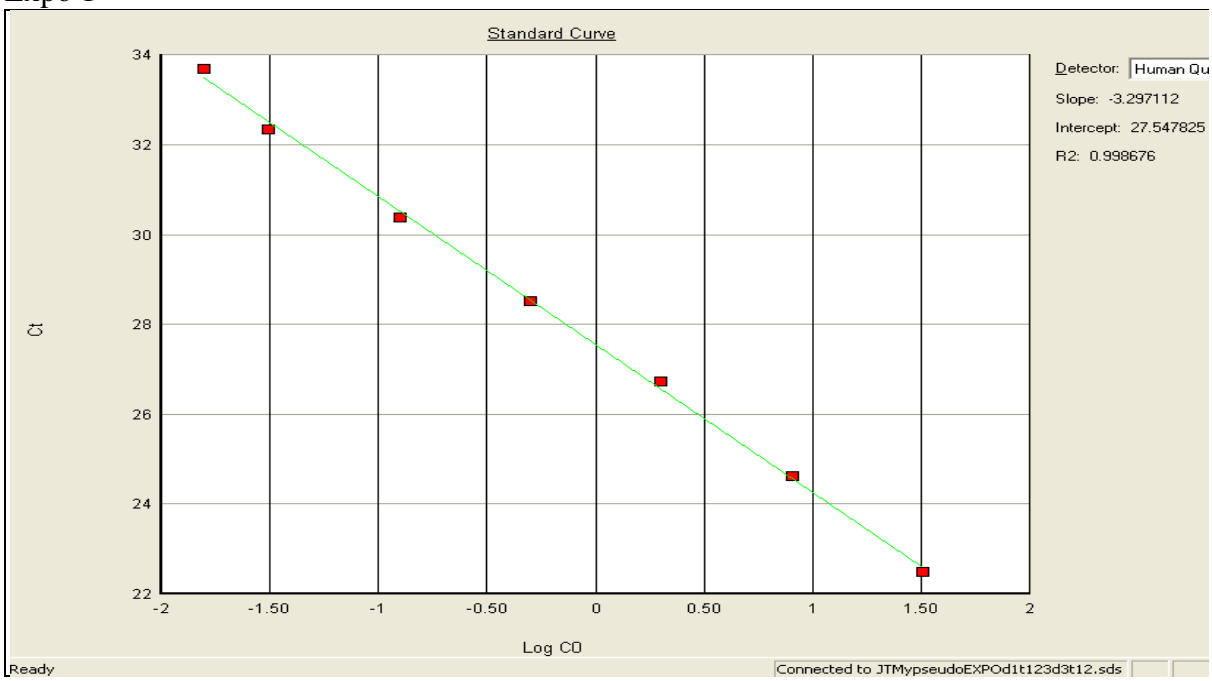


FIGURE B1 **Standard curve for DNA quantification of the prolonged exposure of ambient environment to blood and saliva samples. C_T: threshold cycle of a specified amplification plot that occurs when the fluorescent signal increases beyond the value of the threshold setting.**

Expo 1



Expo 2

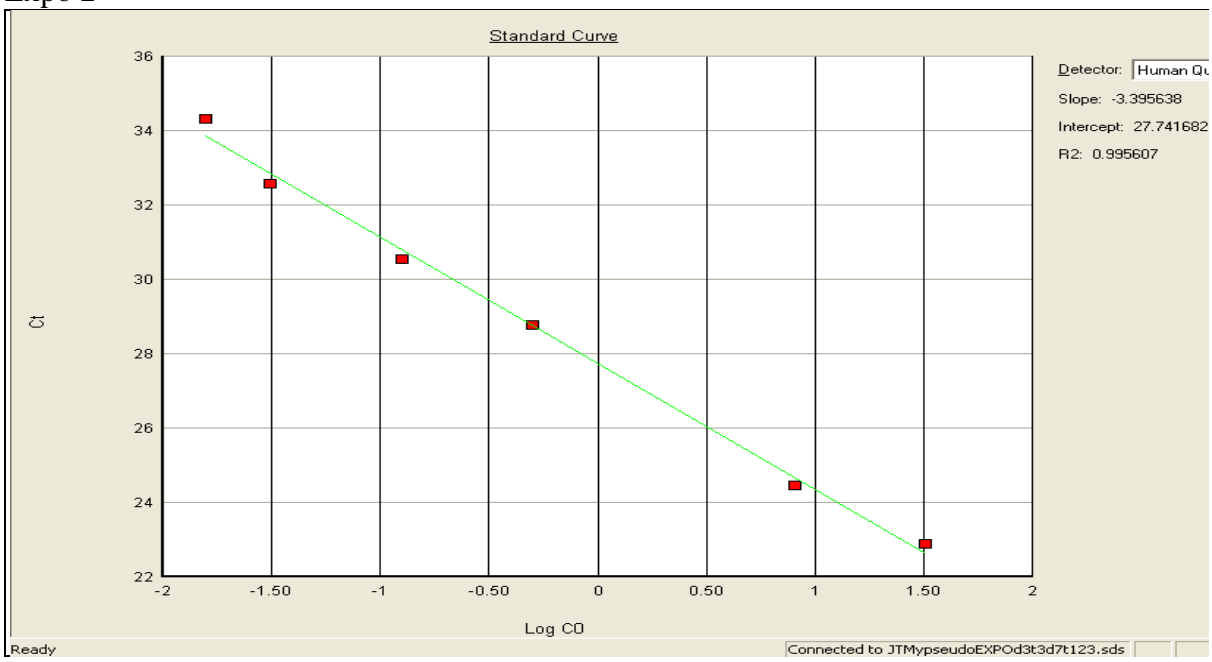
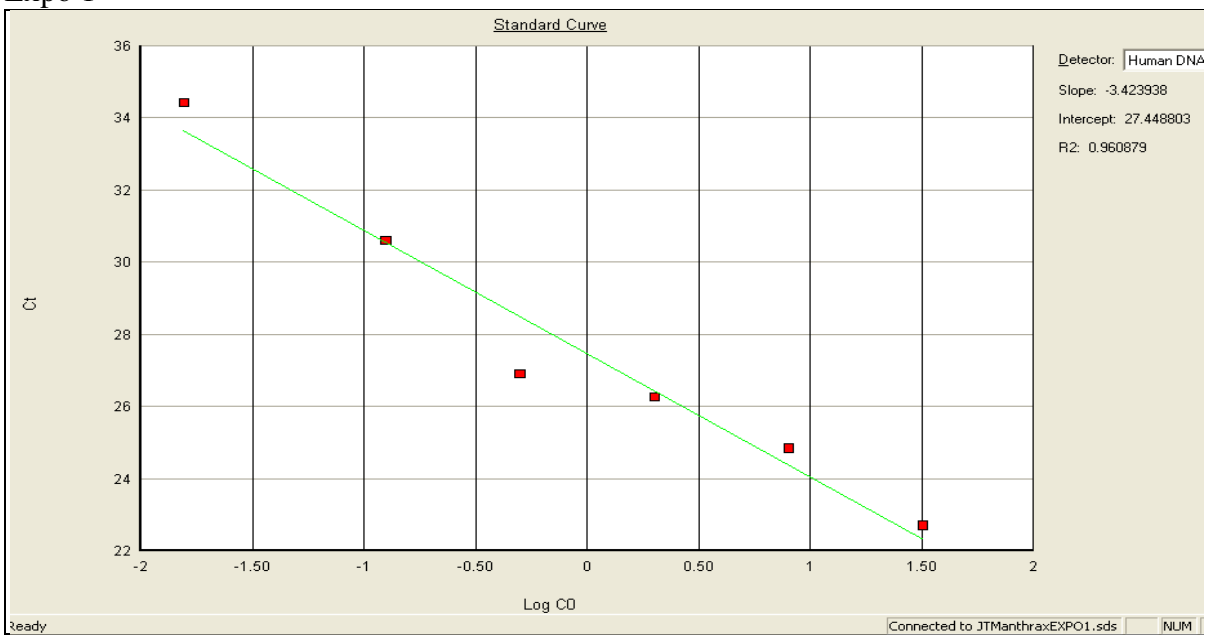


FIGURE B2 **Standard curve for DNA quantification of the prolonged exposure of *Yersinia pseudotuberculosis* to blood and saliva samples. C_T: threshold cycle of a specified amplification plot that occurs when the fluorescent signal increases beyond the value of the threshold setting.**

Expo 1



Expo 2

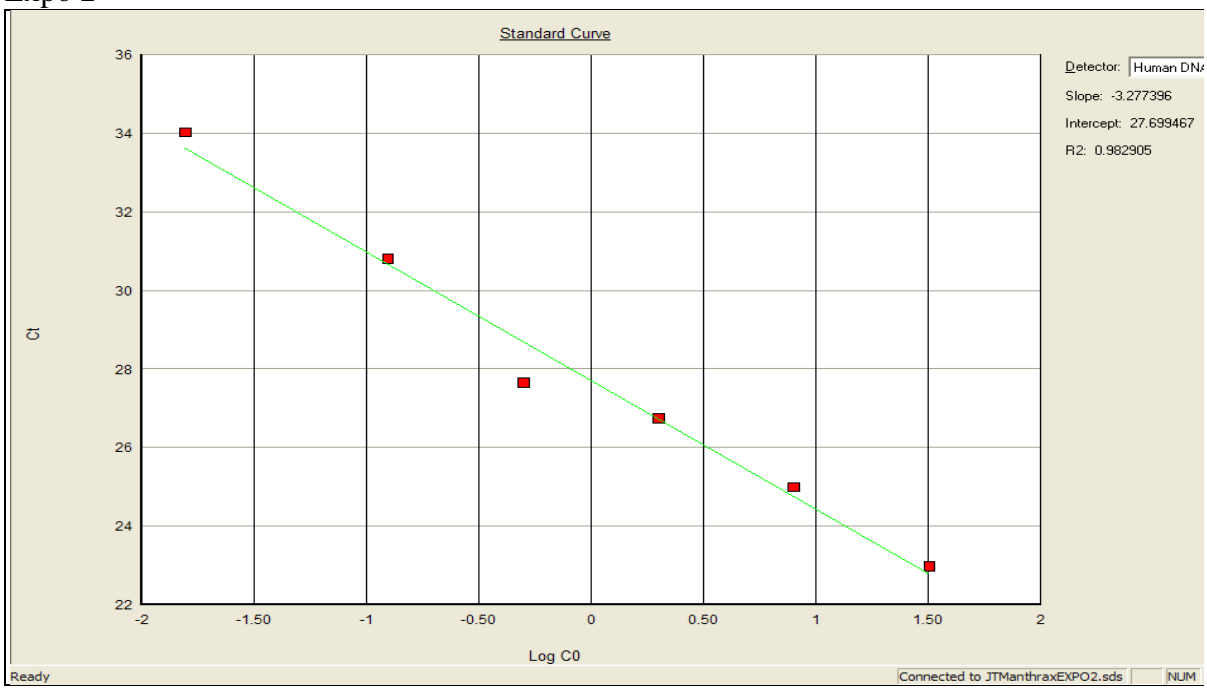
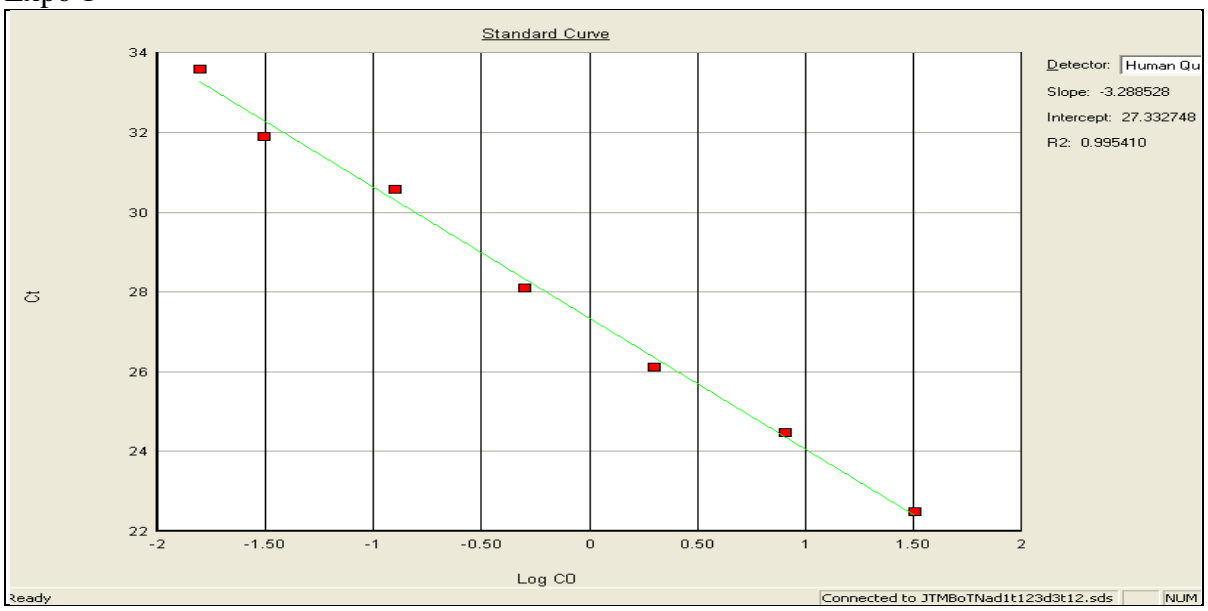


FIGURE B3 **Standard curve for DNA quantification of the prolonged exposure of spores of *Bacillus anthracis* virulent strain to blood and saliva samples. C_T: threshold cycle of a specified amplification plot that occurs when the fluorescent signal increases beyond the value of the threshold setting.**

Expo 1



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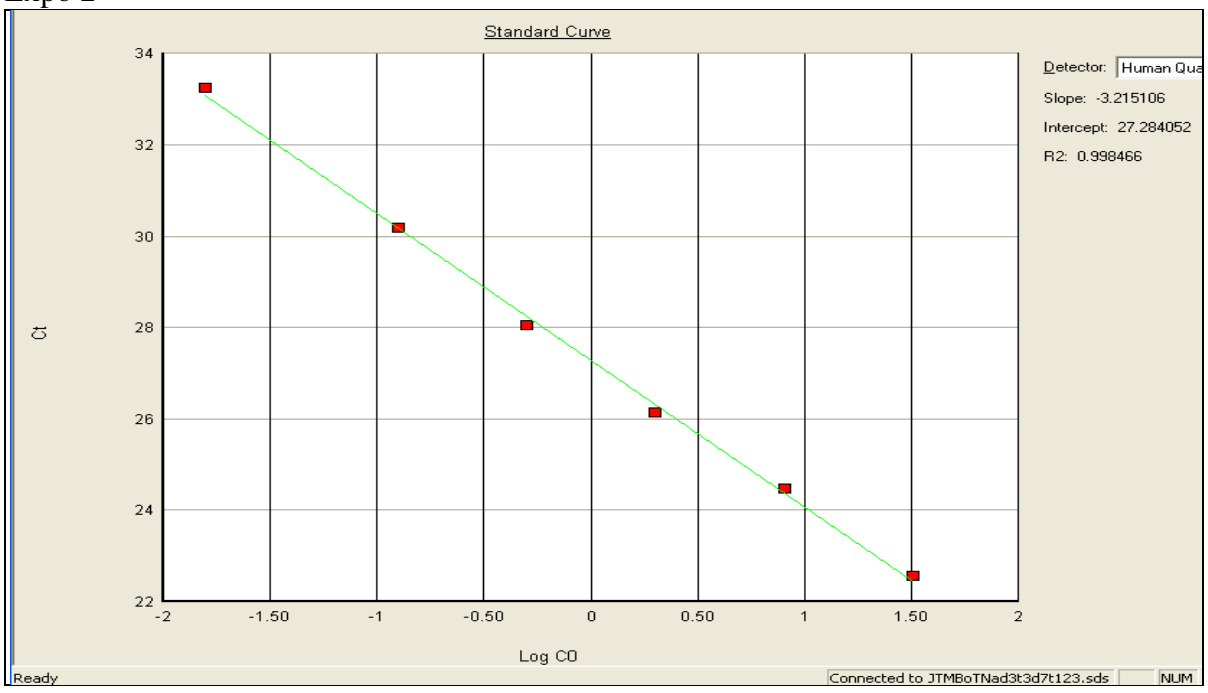
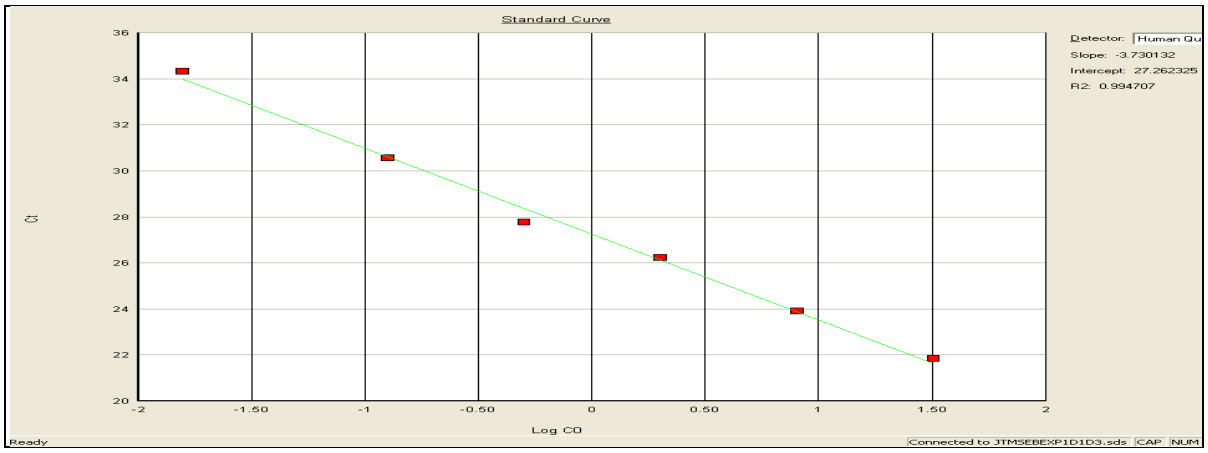
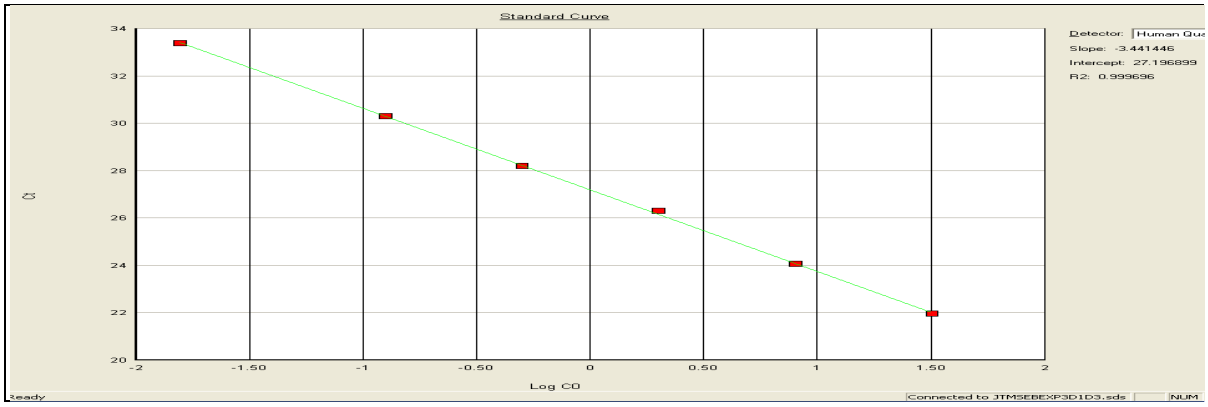


FIGURE B4 **Standard curve for DNA quantification of the prolonged exposure of botulinum toxin A to blood and saliva samples. C_T: threshold cycle of a specified amplification plot that occurs when the fluorescent signal increases beyond the value of the threshold setting.**

Trial 1



Trial 2



Trial 3

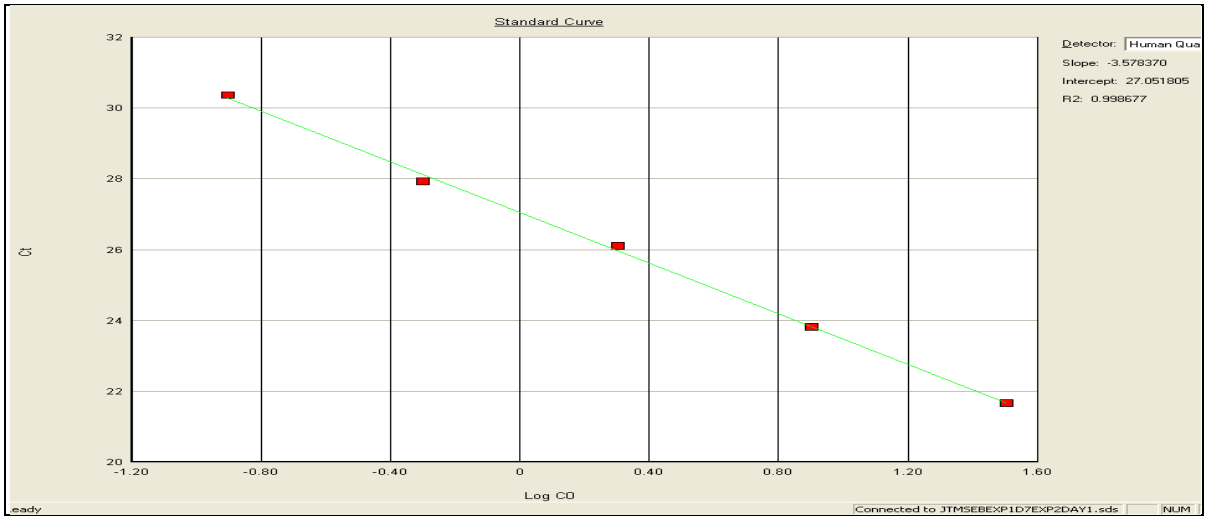
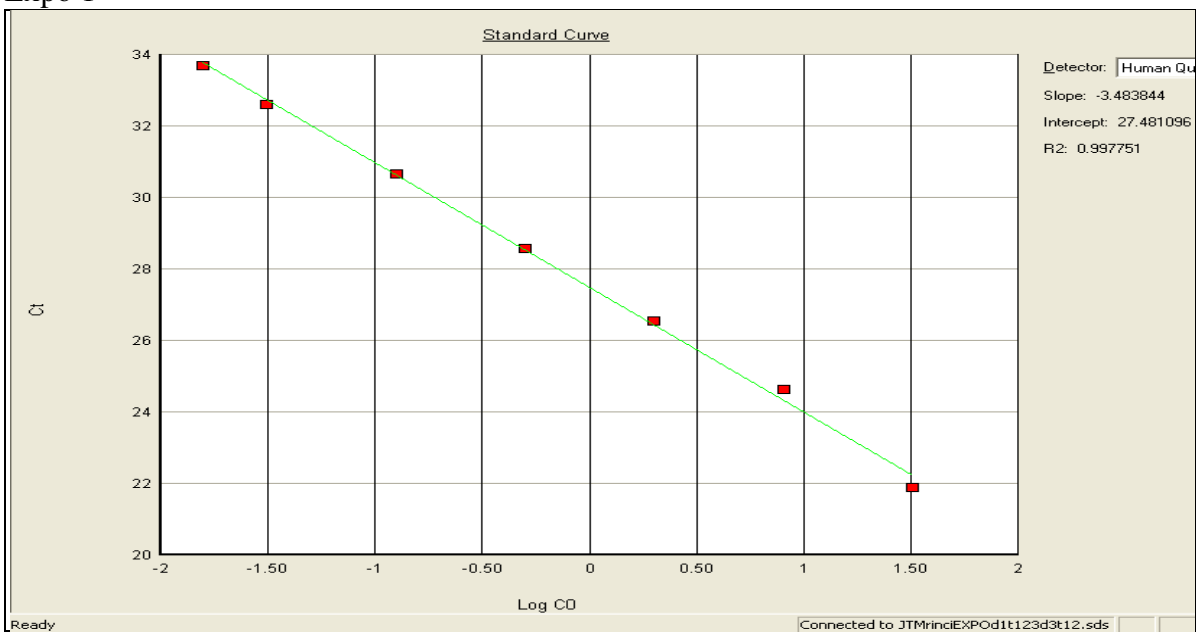


FIGURE B5 **Standard curve for DNA quantification of the prolonged exposure of staphylococcal enterotoxin B to blood and saliva samples. C_T: threshold cycle of a specified amplification plot that occurs when the fluorescent signal increases beyond the value of the threshold setting.**

Expo 1



Expo 2

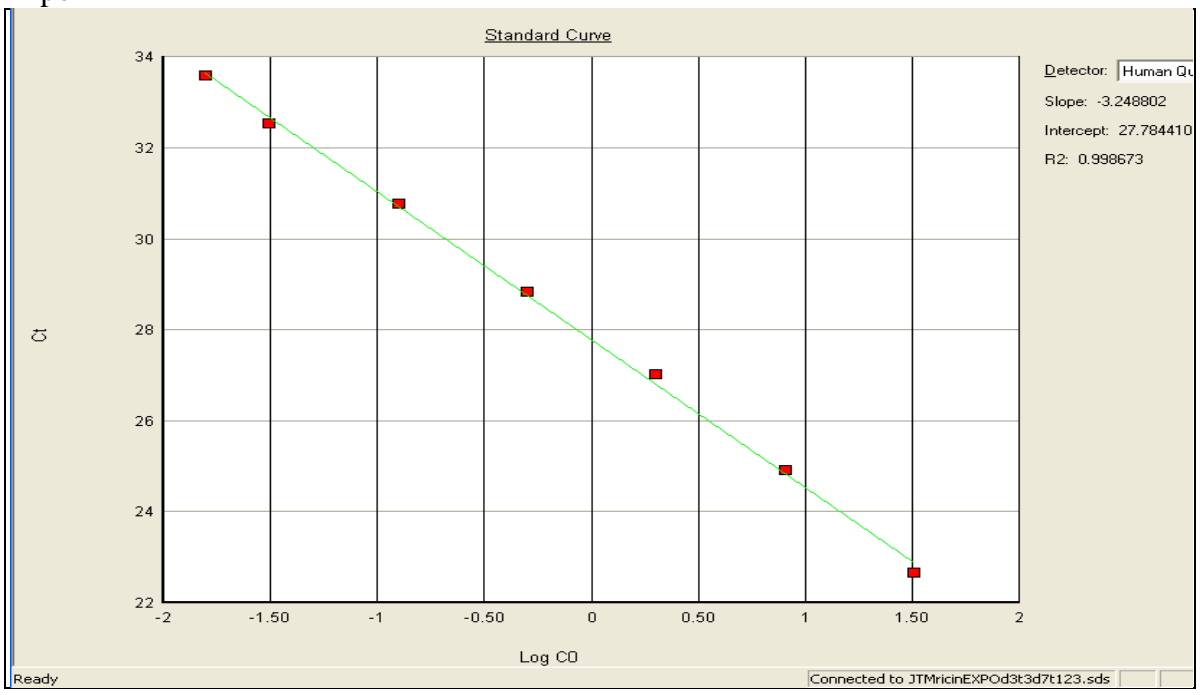


FIGURE B6 **Standard curve for DNA quantification of the prolonged exposure of the recombinant ricin toxoid to blood and saliva samples. C_T: threshold cycle of a specified amplification plot that occurs when the fluorescent signal increases beyond the value of the threshold setting.**

APPENDIX C

COMPLETED STR PROFILES OBTAINED by CAPILLARY ELECTROPHORESIS

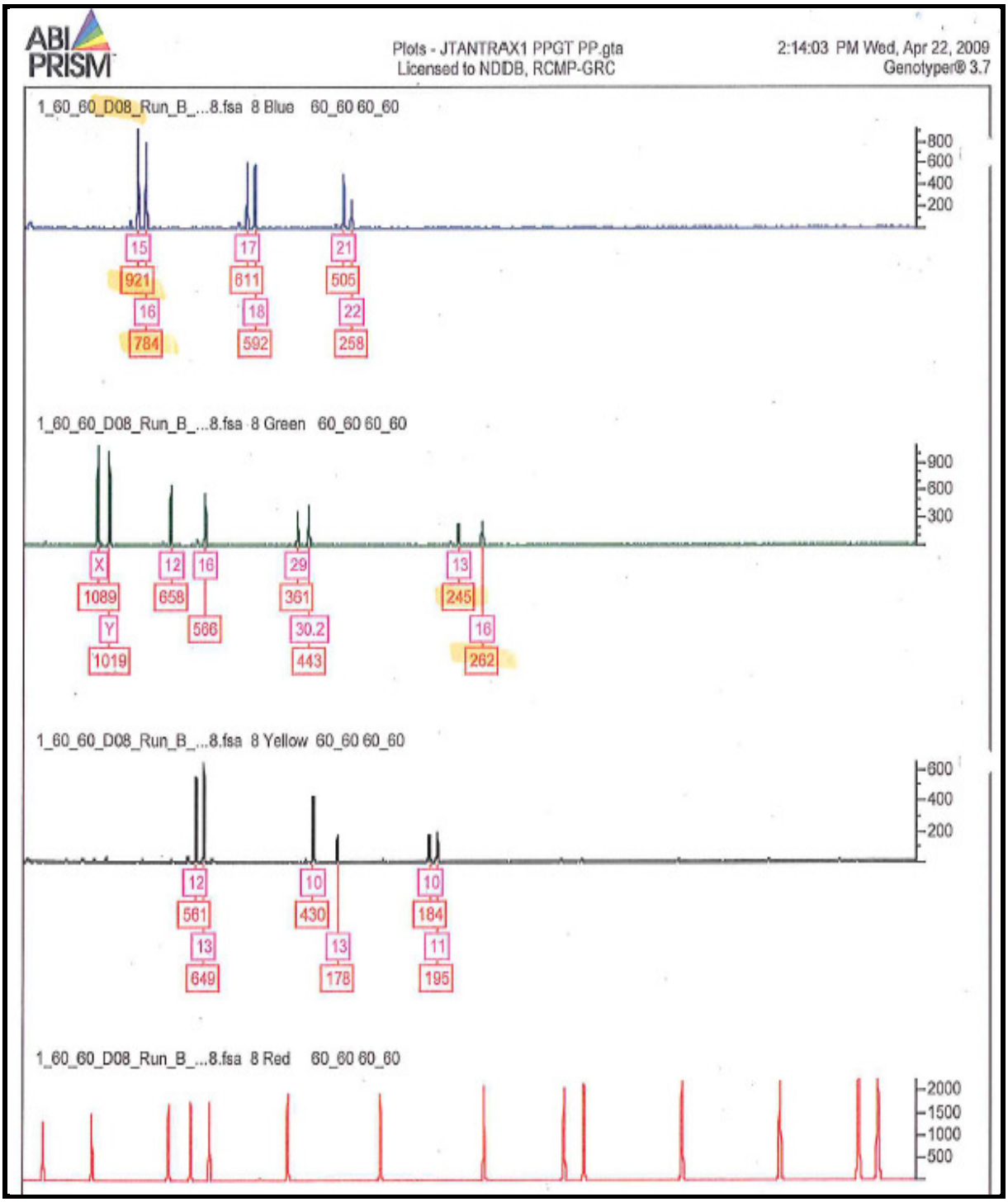


FIGURE C1 **Electropherogram obtained by capillary electrophoresis of a blood sample from a male donor (green line) contaminated with *Bacillus anthracis* spores. Stutter effects are visible in the blue line (first line). The smallest and largest heterozygous STR (D8/D13) are highlighted in orange. Internal standards are represented at the bottom of the electropherogram (red line).**

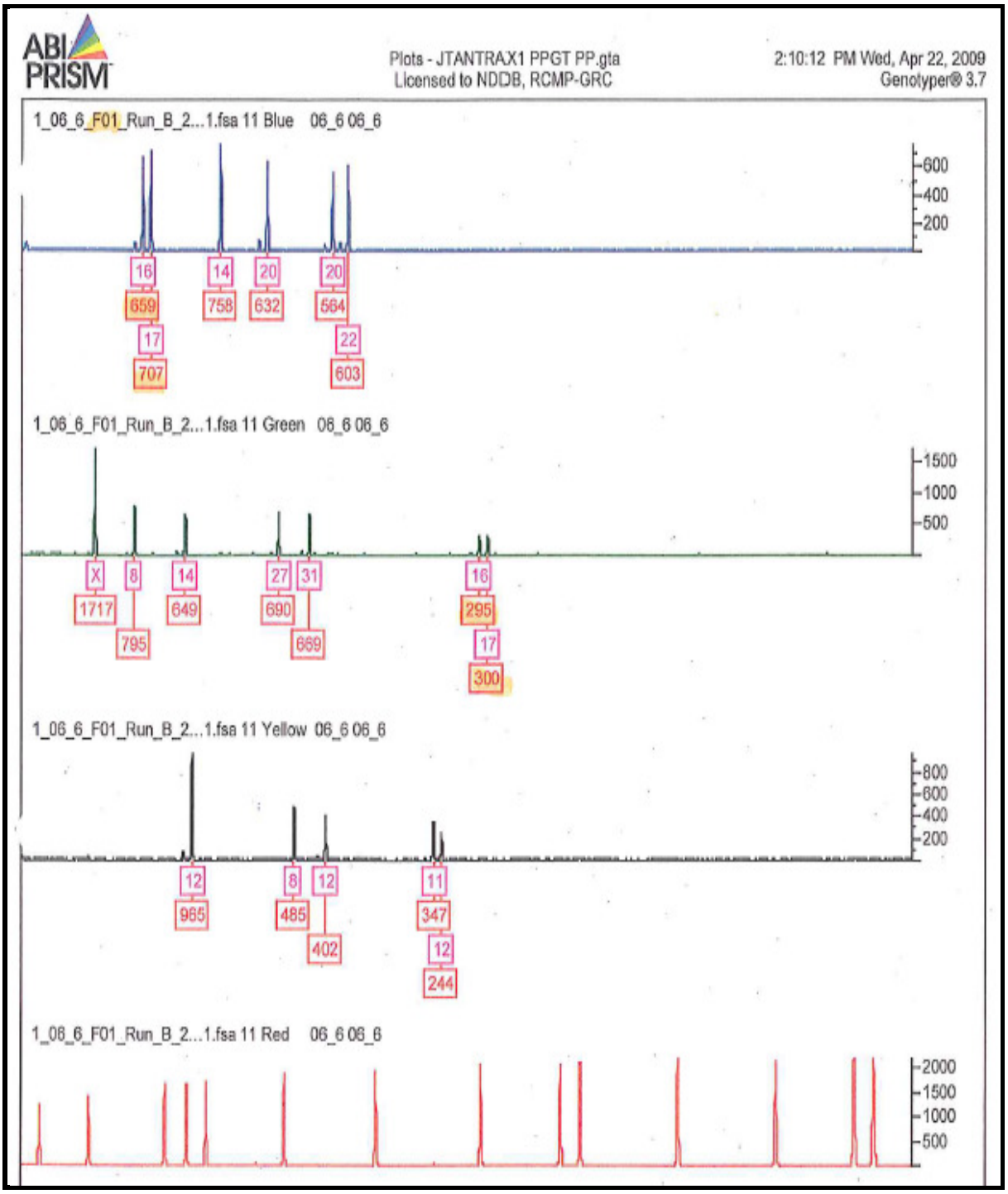


FIGURE C2 **Electropherogram obtained by capillary electrophoresis of a buccal swab sample from a female donor (green line) contaminated with *Bacillus anthracis* spores. Stutter effects are visible (blue line). The smallest and largest heterozygous STR (D8/D13) are highlighted in orange. A homozygous STR is represented by a single peak (yellow line). Internal standards are represented at the bottom of the electropherogram (red line).**