SWEDISH FORUM FOR
BIOPREPAREDNESS DIAGNOSTICS

FBD

PROJECT REPORT

IMPROVED METHODS AND CAPABILITY FOR LABORATORY BIOPREPAREDNESS

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FBD 2016/18

ABSTRACT

This report summarises the activities and outcome of the FBD project *Improved methods and capability for laboratory preparedness*. Based on a gap-analysis, the project has focused on several areas identified to be of certain importance. These focus areas include improvement of diagnostic methods *(Yersinia* and *Brucella*); evaluation of new instruments (rapid test and extraction robot); and training to improve ability (ultrafiltration of water samples).

Title:	Improved methods and capability for laboratory biopreparedness FBD2016/18
Publication number:	MSB1043
ISBN:	978-91-7383-695-1
Project time (duration):	Part 1: 27th of January to 1st of December 2014 Part 2: 27th of January to 26th of November 2015 Part 3: 20th of January to 30th of November 2016
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Financing:	The Project was funded by 2:4 funds for crisis preparedness actions through the Swedish Civil Contingencies Agency, MSB
Layout and print:	Lenanders Grafiska AB, Kalmar, Sweden.

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SCOPE OF THE FBD

The overall aim of the Forum for Biopreparedness Diagnostics (FBD) is to strengthen the capability and capacity to identify microbial high consequence agents (i.e. agents that require biosafety level 3 laboratories) in various sample types and enable the authorities to share the sample load during crisis. To achieve this, the FBD strives to harmonise methods, equipment and quality assurance to ensure that results emanating from the participating authorities are comparable. The multisectoral laboratory network enables diagnostic work applied to different sample types e.g. tissue (human and animal), food, feed, drinking water and environmental samples. FBD is a collaborative effort of four Swedish governmental agencies: the National Food Agency (NFA), the National Veterinary Institute (SVA), the Swedish Defense Research Agency (FOI) and the Public Health Agency of Sweden (PHAS).

FBDs ARBETE

Det övergripande målet med Forum för beredskapsdiagnostik (FBD) är att skapa och förbättra förutsättningar för ett mer effektivt utnyttjande av landets samlade kapacitet och kompetens för diagnostik av biologiska riskklass 3 agens (det vill säga patogener som kräver skyddsnivå 3 laboratorier). Genom sådan samordning ska myndighetslaboratorierna kunna utföra jämförbar och kvalitetssäkrad diagnostik med god kapacitet och uthållighet i händelse av storskalig spridning av allvarlig smitta. Forum för beredskapsdiagnostik (FBD) är ett samarbete mellan fyra svenska myndigheter: Livsmedelsverket, Statens Veterinärmedicinska Anstalt (SVA), Totalförsvarets forskningsinstitut (FOI) och Folkhälsomyndigheten (FOHM), som tillsammans täcker kompetensområdena humanmedicin, veterinärmedicin, foder, livsmedel inklusive dricksvatten, miljöprover samt expertis med avseende på miljöprovtagning och bioforensisk analys.

1. SAMMANFATTNING

Denna rapport sammanfattar de aktiviteter och resultat som utmynnat från FBD-projektet Improved methods and capability for laboratory preparedness. Vid en gap-analys över diagnostisk förmåga som utfördes under våren 2014 identifierades ett flertal områden där det fanns en förmågebrist.

Utifrån en prioriteringslista över områden som identifierades vid gap-analysen har projektet genomfört fem förmågehöjande aktiviteter: förbättring av två diagnostiska metoder för påvisning av *Yersinia* respektive *Brucella*, utvärdering av fältmässigt snabbtest för samtidig detektion av åtta olika agens, marknadsundersökning och utvärdering av extraktionsrobotar samt planerat och utfört en övning i storskalig vattenprovtagning.

Med information om att den extraktionsrobot som i nuläget används av samtliga myndigheter inte längre finns i produktion hos leverantören har projektet utvärderat nya instrument på marknaden i syfte att hitta en likvärdig extraktionsrobot som klarar myndigheternas krav.

Projektet har även planerat och utfört en myndighetsgemensam övning med syfte att förbättra förmåga i vattenprovtagning (ultrafiltrering av vattenprover). Ultrafiltreringsmetoden har sedan tidigare satts upp via FBD på de olika myndigheterna men på grund av att det är en sällananalys, ett antal år har fortlöpt sedan förra övningen och att det har skett en viss omsättning av personal fanns det ett behov av att återigen gemensamt öva metoden.

Sammanfattningsvis har projektet lett till förbättrade och uppdaterade metoder för identifiering, bättre förståelse av begräsningar och möjligheter med snabbtest, träning och övning av förmåga för provtagning av vatten samt inköp av extraktionsrobot som uppfyller kraven för användning på BSL3 laboratorium.

2. GLOSSARY OF TERMS AND ABBREVIATIONS

Biopreparedness	A state of readiness for potential threats to public health, animal health or other emergencies caused by infectious disease or dissemination of pathogenic biological agents.
Biosafety	The application of knowledge, techniques and equipment to prevent personal, laboratory and environmental exposure to potentially infectious agents or biohazards. Biosafety defines the containment conditions under which infectious agents can be safely manipulated.
BSL3	Biosafety Level 3. This biosafety level is applied to facilities where biological agents are handled that cause severe and potentially lethal disease, infect in low doses and can spread via air.
CFU	Colony forming units.
Cq	Cycle of quantification (also known as Ct-threshold cycle).
Effectivity	The fraction of the target DNA that is copied in a PCR cycle.
FOI	Swedish Defence Research Agency[1].
High-consequence agents	Disease-causing microorganisms that require handling at BSL3, according to the work environment authority, and that are likely to cause severe disease or death. Examples are anthracis, tularemia, plague and Q-fever.
IMASS	Integrated Multiplex Assay and Sampling System.
LOD	Limit of detection.
NFA	National Food Agency[2], Sweden.
Pathogenic	Causing or capable of causing disease.
PCR	The polymerase chain reaction (PCR) is a technique used in molecular biology to amplify a single copy or a few copies of a piece of DNA by several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.
PHAS	Public Health Agency of Sweden[3].
PhHV-1	Phocine Herpesvirus 1.
Precision	Includes repeatability and reproducibility.
Real-time PCR	A real-time polymerase chain reaction is a laboratory technique of molecular biology based on PCR. It monitors the amplification of a targeted DNA molecule during the PCR, i.e. in real-time, and not at its end, as in conventional PCR.
Specificity	How well the PCR detects the specific target organism, whereby different species were tested for inclusivity and exclusivity.
SVA	National Veterinary Institute[4] Sweden.

[1] http://www.foi.se
[2] http://www.livsmedelsverket.se
[3] https://www.folkhalsomyndigheten.se
[4] http://www.sva.se

3. BACKGROUND

The Forum for Biopreparedness Diagnostics (FBD) is a collaborative effort between four Swedish governmental institutes – the National Food Agency (NFA), the National Veterinary Institute (SVA), the Public Health Agency of Sweden (PHAS), and the Swedish Defence Research Agency (FOI). The overall objective of the FBD network is to strengthen, through harmonisation, the capability and capacity for diagnostics at the national BSL3 laboratories.

Microbiological preparedness – in particular diagnostics of rare infectious agents that are not routinely used – is challenging. In particular, high-consequence agents can have a serious impact on the community and they require laboratories with higher biosafety levels (BSL3). This entails special demands on method development, education, training, and quality management. The current project set out to identify the participating agencies' most needed improvement of diagnostics from a biopreparedness perspective. Once identified, the project would set up methods to fulfill the needs.

The Armed Forces needs fast and accurate identification of biological threats. Recently BBI Detection in the United Kingdom released an on-site kit called IMASS for detecting eight agents. A task for the current project was to validate the IMASS with regard to specificity and sensitivity.

FBD has earlier developed a method to sample large volumes of water using an ultrafiltration technique. Personnel were trained to use the method and to extract DNA, then subsequently run real-time PCR on the DNA. In order to maintain this capacity, the current project was given the task of organising a practical exercise involving sampling and analysis of environmental water.

4. PROJECT AIM

To strengthen the joint ability for BSL3 diagnostics according to needs identified in gap-analyses performed in 2014 and 2016.

4.1 OBJECTIVES 2014

Part 1: To inventory/identify the participating need of better diagnostics of BSL3 agents.

Part 2: To evaluate the IMASS rapid test developed by BBI Detection in the United Kingdom with regard to specificity and sensitivity for biological agents with focus on the requirements of the Swedish Armed Forces and civilian actors such as the police.

4.2 OBJECTIVES 2015

Part 3: To develop and validate a real-time PCR diagnostic method for *Yersinia pestis*, *Y. pseudotuberculosis,* and *Y. enterocolitica.*

4.3 OBJECTIVES 2016

Part 4: To develop and validate a real-time PCR diagnostic method for identifying all biovars of *Brucella melitensis* and suis.

Part 5: To perform a survey of DNA-extraction robots currently on the market and suitable for a BSL3 facility. To compare the selected robots with the current EZ1 robot, in extraction and analysis of bacterial agents (*Bacillus* and *Francisella*) in relevant matrices.

Part 6: To train the ability to sample and analyse large volumes of environmental water suspected to be contaminated with high-consequence agents using an ultrafiltration method.

5. GAP-ANALYSIS

5.1 AIM

The aim was to inventory the need for new, or improvement of existing, methods for detection of relevant BSL3 agents (bacteria and viruses) and to plan for activities filling the identified gaps.

5.2 METHOD

5.2.1 Gap-analysis

In order to identify the need for new or improved methods at the different agencies a gap-analysis was carried out. The outline of the gap-analysis is presented in figure 1.

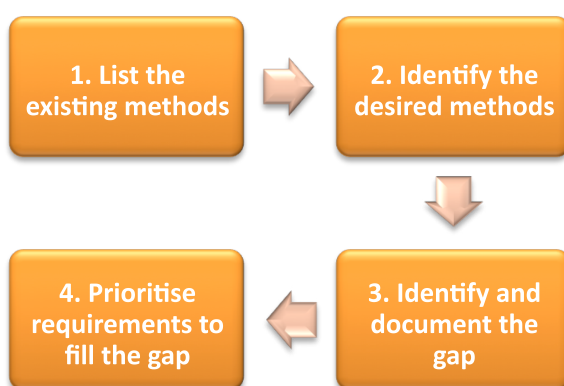

Figure 1. The four steps in the gap-analysis.

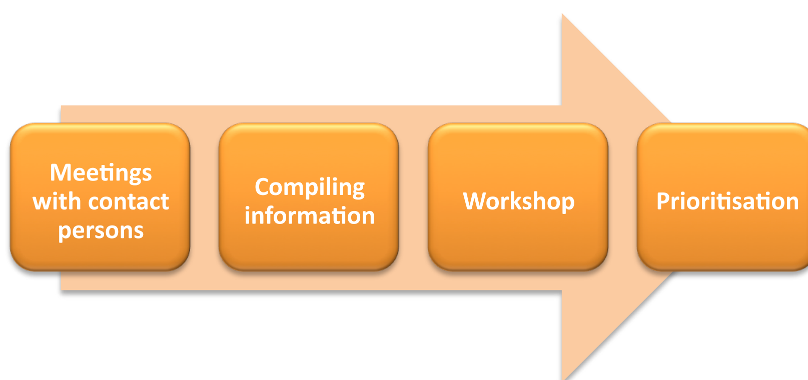

Figure 2. Outline of the work. Initial meetings and compiling of information were performed at each agency; the workshop and prioritisation were done in collaboration by the agencies.

The first two steps of the gap-analysis were carried out at each respective agency where every project member was assigned contact persons who were part of the BSL3 diagnostic preparedness; see figure 2. Together with the contact persons, the project member filled out a table regarding existing and functioning methods, as well as the needs for methods that did not exist or needed updating. Finally, the table included desired capabilities of analyses; see table 1. After the gap-analysis, the results from the different agencies were compiled and presented at a joint workshop in May 2014.

Table 1. Example of the table filled out by each agency.

Methods (existing) / Developed (year)						Method (lacking/need to be updated/harmonised)					Desired ability
Agent	Mole-cular	Cultiva-tion	Immuno-logic	Target	Year	Agent	Mole-cular	Cultiva-tion	Immuno-logic	Comments	
Eg. *B. anthracis*	Real time PCR			pXO1 and 2	09	Eg. *Brucella* spp	Real time PCR			Need to be updated / specificity	Virus
"		Yes, horse blood				*B. canis*			Quick test - poor		
"			No			*Brucella* spp.		Need to be im-proved			

5.2.2 Workshop

The workshop included the project group and the FBD steering committee. The aim of the workshop was to make a priority list of agents and methods based on the compiled gap-analysis. The priority list constituted the base for planning the project for the years 2014 and 2015.

5.2.3 Revision of gap-analysis 2016

In 2016 the agencies renewed the inventory of methods and needs to evaluate if the results from the 2014 inventory were still valid. In this process, some new areas of concern were identified and the priority list for 2016 was revised.

5.3 RESULTS OF GAP-ANALYSIS

5.3.1 Priority list from 2014

5.3.1.1. Activities planned for 2015

Develop and validate a real-time PCR detecting *Yersinia* sp. *enterocolitica, pseudotuberculosis,* and *pestis*.

5.3.2.1 Activities planned for 2016

Transfer knowledge and methods for detecting *Francisella tularensis* to all agencies and with a focus on real-time PCR.

5.3.2 Revised priority list from 2016

5.3.2.1 Activities planned for 2016

Evaluate extraction robots on the market that can replace the EZ1 Advanced (Qiagen), which is no longer in production. The evaluation should look at performance, biosafety, cost, size and availability of reagents.

To plan and perform an exercise to ensure the national capability for retrieval and analysis of water samples suspected to be contaminated with highly pathogenic bacteria.

6. EVALUATION OF THE IMASS

6.1 BACKGROUND

The project intended to evaluate a rapid test system for the detection of bacterial agents and toxins – IMASS (Integrated Multiplex Assay and Sampling System). The focus of the evaluation was the system's specificity and sensitivity and how well it would meet the requirements of the Swedish Armed Forces and civilian actors such as the police. IMASS was developed to provide a simple and fast method of identifying biological agents in the field; it is primarily designed for sampling of surfaces, powders and liquids. The IMASS analyses a single sample for the presence of eight agents simultaneously with an immunoassay strip (figure 3). This kind of rapid test should be regarded as initial screening method to get a fast answer. Upon suspected contamination, the result needs verification from a more conventional method performed in a laboratory. Knowledge of the specificity and sensitivity is crucial in order to assess the usefulness of the rapid test.

6.2 AIM

The aim was to evaluate the IMASS developed by BBI Detection in the United Kingdom with focus on specificity and sensitivity for detection of bacterial and toxin agents.

6.3 METHOD

The system consists of an integrated sponge and eight lateral flow immunoassays that can detect eight biological agents simultaneously: five bacterial and three toxins (*Bacillus anthracis, F. tularensis, Y. pestis, Burkholderia mallei, Brucella* spp., Ricin, *Botulinum* Toxin A and B, and *Staphylococcal* Enterotoxin B); see figure 3. In this project we focused on three bacterial agents and two toxins (*B. anthracis*, F. *tularensis*, *Y. pestis*, Ricin, and *Botulinum* Toxin A). The tests were analysed for specificity and sensitivity. Five strain variants and five strains of species closely related to the target species were included for each bacterial agent tested. For the toxins, in addition to pure samples, agglutinin was added to the test tube in order to evaluate cross-reactivity. Each test was visually read after two and 15 minutes and scored by comparing the test strip with a visual scoring card provided by BBI Detection. Studies were performed both on single immunoassay strips as well as on the complete system (shown in figure 3).

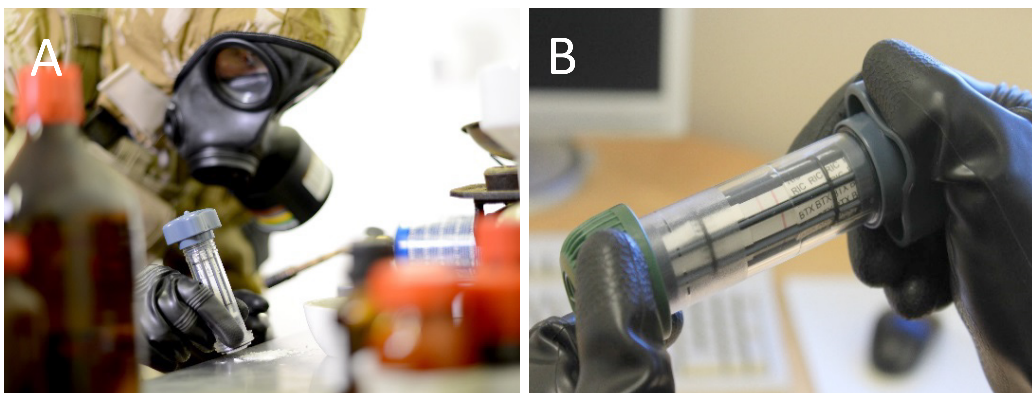

Figure 3. **Panel A** Sampling of powder from a benchtop surface with the IMASS (Integrated Multiplex Assay and Sampling System). An integrated sponge in one end of the IMASS is used to samples the powder.
Panel B Reading of results, which appear as red lines on the immunoassay strips. Images are © BBI Detection Ltd.

6.4 RESULTS OF EVALUATION OF THE IMASS

The limit of detection (LOD) was determined using the most responsive bacterial strain with the highest intensity of signal in the specificity test. The results are summarised in table 2.

Table 2. The results from the evaluation are summarised in this table. (*) The test was negative with the concentrations tested: 10^7 CFU/ml for *B. anthracis*, 10^8 CFU/ml for *Y. pestis,* and 100 ng/ml for *botulinum* toxin A. However, the complete system was tested with the same volume and concentration as the single test-strip meaning that the sample was distributed over the eight strips, thereby reducing the total amount of the agent on each strip.

Target	Specificity (inclusivity)Number of strains or toxins detected/ number tested	Cross-reactivity (exclusivity)	LOD strips Bacteria CFU/ml Toxins ng/ml	LOD IMASS Bacteria CFU/ml Toxins ng/ml	Comments
B. anthracis (veg. cells)	3/5	No	107	-*	Missed two modified strains lacking one virulence plasmid each.
F. tularensis	5/5	No	106	106	
Y. pestis	5/5	No	107	-*	
ricin	1/1	Agglutinin	1	50	
botulinum toxin A	2/2	No	5	-*	

For further information on the method and the results; see evaluation report, appendix 12.1.

6.5 DISCUSSION

The IMASS is a method with good specificity for the bacterial targets and no cross-reactivity to the closely related species included in this evaluation. However, for the two toxins, ricin and *botulinum* toxin A, cross-reactivity was observed for agglutinin in the ricin test. Agglutinin, which is less toxic than ricin, is often present in "dirty samples". The protein is highly homologous to ricin which makes it technically difficult to discriminate between the two proteins by immunoassays. For *botulinum* toxin A, two preparations of the toxin were tested – a complexed, naturally occurring variant and a recombinant version containing only the toxin in its pure form. Except for *botulinum* toxin A, all tests were done with pure cultures or purified toxin meaning that cross-reactivity to components in common matrices cannot be excluded without further evaluation.

Notably, the IMASS could not detect two modified *B. anthracis* strains, each lacking one of the virulence plasmids (pXO1 or pXO2). Given that bacterial strains can alter their genetic content by adding or deleting DNA/plasmids or that a strain can be deliberately genetically manipulated to change its characteristics e.g. virulence, this is something that must be taken into account.

The LODs for ricin and *botulinum* toxin A were very good for the single test strips and the *botulinum* toxin A was detected both in its complex and pure form. However, the complete system requires high bacterial- and toxin concentrations. Given that the sample needs to be distributed over eight strips, this requires larger sample volumes; see the results for LOD IMASS in table 2. However, the purpose of the IMASS is to sample "white powder" incidents where the concentration of a putative B-agent would presumably be relatively high.

7. A REAL-TIME PCR ASSAY FOR DETECTION OF YERSINIA SPECIES

7.1 BACKGROUND

Plague is an infectious disease that affects animals and humans. It is caused by the bacterium *Yersinia pestis*. The bacterium is found in rodents and their fleas and is endemic in many areas of the world. Pneumonic plague occurs when *Y. pestis* infects the lungs. This type of plague can spread from person to person through the air and, left untreated, has a mortality rate approaching 100 %[5,6]. Hence, early treatment of pneumonic plague is essential. To reduce the risk of death, antibiotics must be given within 24 hours of first symptoms. Bubonic plague, which is the most common form of plague, occurs when an infected flea bites a person. Patients develop swollen, tender lymph glands (called buboes). Septicemic plague occurs when plague bacteria multiply in the blood. It can be a complication of pneumonic or bubonic plague or it can occur as a primary infection. Bubonic and septicemic plague usually do not spread from person to person.

Yersiniosis caused by *Y. pseudotuberculosis* and *Y. enterocolitica* can affect both humans and animals. In humans, these infections are often food borne (undercooked meat, vegetables or un-pasteurized dairy products) with gastroenteric symptoms such as diarrhea, vomiting, abdominal pain and fever. *Y. pseudotuberculosis*, as the name implies, can cause tuberculosis-like symptoms in animals like granulomas in the spleen, liver and lymph nodes. In humans, it can cause a disease known as Far East scarlet-like fever, with symptoms sometimes mimicking appendicitis (fever and right-sided abdominal pain). In cases involving immunocompromised patients, antibiotics may be necessary for clearance of the bacteria. *Y. enterocolitica* is found all over the world and is common in pig tonsils and feces. Also dogs and cats can be carriers of the bacterium. Humans are often affected by eating raw or undercooked pork contaminated with the bacterium. *Yersinia* can also be found in water and food that has been in contact with contaminated water.

The gap-analysis performed in May 2014 (see section 5 Gap-analysis) identified the need for a PCR method detecting all three pathogenic *Yersinia* species. Current PCR methods detect only *Y. enterocolitica* strains encoding the virulence plasmid. However, *Y. enterocolitica* strains lacking the virulence plasmid can still cause disease in immunocompromised people[7]. Because of this, there was an interest to for a PCR method to identify *Y. enterocolitica* strains lacking the virulence plasmid.

7.2 AIM

In this study, we aimed to establish a new real-time PCR assay for the identification of three species of *Yersinia*: *Y. pestis*, *Y. pseudotuberculosis,* and *Y. enterocolitica*.

[5] Kool, J. L., & Weinstein, R. A. (2005). Risk of person-to-person transmission of pneumonic plague. *Clinical Infectious Diseases*, 40(8), 1166-1172.
[6] Titball, R. W., & Williamson, E. D. (2001). Vaccination against bubonic and pneumonic plague. *Vaccine*, 19(30), 4175-4184.
[7] Sabina et al., (2011) Yersinia enterocolitica: Mode of Transmission, Molecular Insights of Virulence, and Pathogenesis of Infection. Journal of pahtogens (2011) Article ID 429069.

7.3 METHOD

The pathogenic *Yersinia* species –*Y. pestis, Y. pseudotuberculosis,* and *Y. enterocolitica* – are at diametrically opposite ends of the *Yersinia* genus tree[8] (figure 4); therefore it has been difficult to establish a real-time PCR that detects the three species simultaneously. Bioinformatical studies identified the gene encoding an efflux transporter – outer membrane factor (locus tag CH49_4148) – as a suitable target. PCR-systems were designed using the SMI primer software developed at PHAS.

For validation of the method, the FBD005 protocol[9] was used. The study included a collection of 11 *Y. pestis* strains, 25 *Y. pseudotuberculosis* strains, 59 *Y. enterocolitica* strains, and 58 other bacteria of which 19 were near-neighbouring strains. Some of the strains are classified as BSL3 and were therefore cultured and handled in BSL3 laboratories accordingly. The strains were cultured onto agar plates and incubated at 26 °C overnight. Subsequently, DNA was extracted with an extraction robot (EZ1 Advanced, Qiagen using EZ1DNA Tissue Kit). The DNA concentration was measured (Qubit Fluorometer) and diluted to 2 ng/µL for the PCR. Control-DNA from target strains was used in serial dilutions to evaluate the performance of the PCR.

Figure 4. The phylogeny of the genus *Yersinia*. The pathogenic species are marked with red circles. pYV indicates when the Yersinia virulence plasmid appeared in the different lineages.
Figure depicted from Reuter et al., (2014) Parallel independent evolution of pathogenicity within the genus *Yersinia*. PNAS 111(18):6768-6773.

[8] Reuter et al., (2014) Parallel independent evolution of pathogenicity within the genus *Yersinia*. *PNAS* 111(18):6768-6773.
[9] FBD 005-2 Valideringsmanual för kvalitativ realtids PCR-analys för detektion av bakterier.

7.4 RESULTS OF THE VALIDATION

Y. enterocolitica is distantly related to *Y. pestis* and *Y. pseudotuberculosis* making it difficult to design one primer pair to identify all three species of interest while excluding the many non-pathogenic species within the genus (figure 4). Therefore, two primer pairs were designed, one for *Y. enterocolitica* and one detecting both *Y. psudotuberculosis* and *Y. pestis* (table 3).

Table 3. Primers and probes.

Target (species)	Oligo name	Sequence
Y. enterocolitica	Ye_gen_F	CGGTAYCTGTTGGGCTTTCCT
Y. enterocolitica	Ye_gen_R	CATTAGCCGATTTCAATTTATGCTC
Y. pestis/ Y. pseudotuberculosis	Yp_Ypt_gen_F	TGTACCCGTTGGGCTTTCCT
Y. pestis/ Y. pseudotuberculosis	Yp_Ypt_gen_R	TGGCCGATTTCAGTTTATGCTC
Y. pestis/Y. pseudotuberculosis/Y. enterocolitica	Y_gen_FAM-MGB	CTGATGTGTTGTTGAACCG

The tested strains were detected correctly by the two real-time PCR assays. Thus both specificity and sensitivity were 100 %; see table 4. The validation was divided into two parts. Part 1 (specificity) was performed at all agencies where each laboratory analysed one species. Part 2 (precision) was performed at PHAS and SVA with two different species, *Y. pestis* and *Y. enterocolitica*. The complete results of the validation are summarised in the validation report; see appendix 12.2.

Table 4. Results of the validation. For the inclusivity, all of the 87 strains of *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* tested were detected with the PCR-assay. For the exclusivity, none of the 56 strains of other species, including near-neighbours, was detected.

	Summary	
Specificity, inclusivity (%)	100 %	
Specificity, exclusivity (%)	100 %	
	PHAS *(Y. pestis)*	**SVA *(Y. enterocolitica)***
Efficiency (%) and confidence interval	102 % +/- 0.01190	105 % +/- 0.00072
LOD (GE/µL)	6.25	6.25
Precision, repeatability	1.30	2.32
Robustness	Evaluated	Evaluated

Interestingly, during validation, two *Y. enterocolitica* strains were not detected with the new assay whereas two near-neighbouring strains (*Y. kristensenii* and *Y. fredriksenii*) were. In order to confirm the classification, the four strains were analysed by MALDI-TOF (Bruker Daltonics Microflex LT). The characterisation of the strains showed that the previous classification was wrong; see table 5 below.

Table 5. Results from the MALDI-TOF analysis.

Strains	Previous classification	New classification
1	*Y. enterocolitica*	*Y. massiliensis*
2	*Y. enterocolitica*	*Y. kristensenii*
2126	*Y. kristensenii*	*Y. enterocolitica*
979019	*Y. fredriksenii*	*Y. enterocolitica*

7.5 DISCUSSION

The specificity, sensitivity and efficiency of the developed PCR-assay were very good. Thus the new assay fulfilled the criteria for implementation in FBD, and it has been implemented at FOI. Since the definition of non-pathogenic *Y. enterocolitica* strains is very diffuse, the PCR was designed to detect all strains of this species. For *Y. pseudotuberculosis* there are two unusual serotypes (O: 11 and O: 12). In previous studies these have been difficult to detect because they differ from other serotypes[10]. It is unclear whether the new PCR can identify these serotypes or not.

A lesson learned during this project is that it is important to know the source and history of the reference strains. New and better methods can reveal previous mistakes in classification. Ideally, to allow correct interpretations of results, both inclusivity and exclusivity strain panels need to accurately represent the best possible genetic diversity of the species or lineage under investigation. Thus they need to represent the best available range of geographical and temporal occurrences of the organism, as well as strains originating from different environments and hosts. In addition, pathogens are dynamic and panels must be continually updated with isolates from contemporary outbreaks. Availability of accurate strain reference panels is always a challenging and difficult task. As new strains and genome sequences become available, methods need to be continuously tested and evaluated.

8. A REAL-TIME PCR ASSAY FOR DETECTION OF BRUCELLA SPECIES

8.1 BACKGROUND

Brucellosis is a widespread zoonosis that infects mainly cattle, sheep, goats, and pigs, despite ongoing eradication programs. Furthermore, it can also infect humans, with more than 500.000 human cases reported worldwide annually. It is considered to be a febrile illness with localised bone and tissue infection, or multi-organ disease. It also leads to considerable financial losses in animal husbandry due to abortion and fertility problems in cattle, sheep, and goats. Transmission to humans occurs through different channels: ingestion of unpasteurized milk and dairy products thereof; direct contact with infected animal tissues; or accidental ingestion, inhalation or injection of cultured *Brucella*. Most Brucellosis cases occur around the Mediterranean basin (Portugal, Spain, Southern France, Italy, Greece, Turkey, and North Africa). *Brucella* comprises six classical species (*B. abortus, B. canis, B. melitensis, B. neotomae, B. ovis,* and *B. suis*) and five novel species (*B. ceti, B. microti, B. inopinata, B. papionis,* and *B. pinnipedialis*). *B. melitensis* is recognised as the predominant species associated with human outbreaks worldwide.

The species of the genus *Brucella* can be distinguished on the basis of phenotype, genotype and preferred host. All cases in Sweden originate from abroad. The incidence in Sweden is between 1-20 reported cases per year (Sminet2, Swedish National Surveillance System). The gold standard method for its diagnosis is the isolation of the bacteria from clinical samples via blood cultures and identification by classical microbiological tube testing. *Brucella* grows slowly; visible colonies usually appear after 3-4 days, but it can take more than two weeks to obtain a definitive result. The application of DNA based methods to *Brucella* diagnostics is challenging because all *Brucella* species have a very high degree of genetic homology (up to 99.9 %) as shown by whole genome sequencing of *B. abortus, B. melitensis*, and *B. suis*.

[10] Lambertz, Nilsson and Hallanvuo, (2008) TaqMan-based real-time PCR method for detection of *Yersinia pseudotuberculosis* in food. *Applied and Environmental Microbiology* 74(20):6465-6469

8.2 AIM

The aim was to develop a specific real-time PCR for the detection of all biovars of *B. melitensis* and *B. suis*, and thereby increase capability for laboratory preparedness.

8.3 METHOD

The genomes of *B. ceti*, *B. inopinata*, *B. neotomae*, and *B. suis* biovar 4 were sequenced and analysed, due to the lack of such genome data in the public available databases at the start of the project. Data from all complete genome sequences of all type strains and reference strains were included in the design of primers and probes for the real-time PCR assay of this study. For validation of the method, the FBD005 protocol[11] was used. All available complete genomes of *Brucella* (n=96), including all complete genomes of *B. melitensis* (n=17), were used in the design of primers and probes; see table 6. A two-basepair deletion, highly specific for *B. melitensis*, was found in chromosome 1 (acetyl-CoA acetyltransferase domain protein). Primers were designed flanking this deletion and a short probe with an MGB modifier was placed over the deletion. An extraction robot (EZ1 Advanced, Qiagen using EZ1DNA Tissue Kit) was used for bacterial DNA extraction from a collection of 31 *Brucella* sp. reference strains, 120 *B. melitensis* human clinical isolates, and 46 other bacterial strains for the exclusivity test. Control-DNA with a dilution series was used to check the performance of the PCR.

Table 6. Primers and probes.

Oligo name	Sequence
melitensis_EAF1	AAGGTCAGTTCCATGCGCG
melitensis_EAR1	TCACGCTGGCCCCTTTG
melitensis_EAP1	FAM-TGTTGAAGGTAAGCGTGTTCTGATT-MGB
melitensis_EAF2	GGACAATCGTTATCGGCGAT
melitensis_EAR2	GCCCTTGCCTGTGATGATAAC
melitensis_EAP2	FAM-CGATCCGCAGGCGTTTCGTGG-TAMRA
melitensis_EAF3	GCTCGACACAAAGGGCCA
melitensis_EAR3	CAAGCGTGGTCTGGCGA
melitensis_EAP3	FAM-CCGCCGAGATACAAA-MGB
Reverse probe melitensis_EAP4	FAM-ATCTCGGCGGCGGC-MGB
Shorter probe melitensis_EAP5	FAM-CCGAGATACAAA-MGB
suis_EAF1	GAGCCGGGCAATGCGATT
suis_EAR1	GAAACCGACCAGCCCGTT
suis_EAP1	FAM-ACCCGGCGCGCATTCCGGCGGCACT-TAMRA
suis_EA_F2	TGGGTTCGCAAACCATGC
suis_EA_R2	GGGCTGGATCGGCCACTA
suis_EA_P2	FAM-ATCACAAAAGCGCAAAGATCACACCGT-TAMRA
suis_EAF3	GAAATCGGCATGATGCAGCT
suis_EAR3	CGCTGTTCTTCAGATCGACCT
suis_EAP3	CTGCCGAAGACTATCT
suis_EAF4	GGGAACGCAACCACCGG
suis_EAR4	GCCATTTCCATTGTCATCGGA
suis_EAP4	FAM- CAGCGATAACAGCG-MGB
suis_F5	GCCAAATATCCATGCGGGAAG
suis_R5	TGGGCATTCTCTACGGTGTG
suis_P5	FAM-TTGCGCTTTTGTGATCTTTGCGCTTTATGG-TAMRA

[8] FBD 005-2 Valideringsmanual för kvalitativ realtids PCR-analys för detektion av bakterier.

8.4 RESULTS OF THE VALIDATION

The real-time PCR developed for *B. melitensis* showed very good specificity and sensitivity. It has therefore been implemented at PHAS for diagnosis of *Brucella* sp.[1]; see table 7. This method is also used by SVA and FOI for identification of *Brucella* sp. in samples. For *B. suis*, an already published real-time PCR was implemented at SVA and PHAS[2].

Table 7. Results of the validation. For the inclusivity, six *B. melitensis* strains and 120 *B. melitensis* human clinical isolates were detected with the PCR-assay. For the exclusivity, none of the 76 strains of other species, including near-neighbours, was detected. For B. suis, nine strains were included in the inclusivity test, of which eight strains were detected with the PCR-assay. For the exclusivity, 73 strains of other species, including near-neighbours, were included and none was detected.

Summary		
	B. melitensis	***B. suis***
Specificity, inclusivity (%)	100 %	89 %
Specificity, exclusivity (%)	100 %	100 %
Efficiency (%) and confidence interval	112 % +/- 0.993	113 % +/- 0.99
LOD (GE/reaction)	6.25	6.25
Precision, repeatability	0.61	2.08
Robustness	Risk assessment	Risk assessment

The complete results of the validation are summarised in the validation reports; see appendix 12.3.

8.5 DISCUSSION

The high genetic similarity of different *Brucella* species makes the design of a species-specific, real-time PCR assays difficult. In total, five different primer and probe combinations were tested and evaluated for *B. melitensis* and *B. suis* to obtain a primer and probe combination sensitive and specific enough. The PCR method for *B. melitensis* has not only good sensitivity and specificity, but also good precision, both in terms of repeatability and reproducibility. It has also been used in a ring-trial with accurate results. The developed real-time PCR assay for *Brucella melitensis* is used for diagnostic purposes at the PHAS, and is also implemented at SVA and FOI.

Unfortunately we did not succeed with a real-time PCR that could detect all five biovars of *B. suis*. The real-time PCR validated for *B. suis* is robust but detects only four biovars (biovars 1- 4). It has 100 % specificity for these four but does not detect biovar five, which is why the inclusivity value is 89 %. The method has good sensitivity. Even though all species of the genus *Brucella* are genetically very similar, no false positive results were obtained regardless at which institute the real-time PCR was done. This indicates that the method is robust. This PCR is implemented at all agencies.

[12] Kaden, Alm, Ferrari and Wahab, (2016) A Novel Real-Time PCR assay for specific detection of Brucella melitensis, submitted.
[13] Hänsel et al., (2015) Novel real-time PCR detection assay for Brucella suis, Veterinary Record Open 2015:2.

9. EVALUATION OF DNA EXTRACTION ROBOTS FOR USE IN A BSL3 LABORATORY

9.1 BACKGROUND

An automated DNA-extraction robot, BioRobot EZ1 (Qiagen, Hilden, Germany), was thoroughly evaluated within the FBD for use in a BSL3 laboratory in 2009[14]. Automated DNA-extraction is safer than manual DNA-extraction and more samples can often be processed in the same amount of time. The current model of the Biorobot EZ1, the EZ1 Advanced, is used at the BSL3 laboratories at all four agencies since the initial evaluation in 2009. The EZ1 is able to extract bacterial DNA compatible with PCR-analysis from many different matrices (e.g. blood, food and feed), making it suitable for use by all FBD agencies. Other benefits of the EZ1 Advanced include: easy-to-use; no manual dispensation of reagents (strips are pre-portioned); internal UV-treatment for decontamination; and a relatively small size which allows the robot to fit in a biosafety cabinet (hence avoiding the risk of spread of contaminated aerosol within the lab). It can perform six extractions per run, with each run taking about 15 minutes.

However, the gap-analysis revision in January 2016 identified the need to find a replacement for the EZ1 Advanced since the manufacturer has discontinued it and it will be difficult to obtain spare parts for it. The kits needed for DNA-extraction are still available since the larger version of the instrument – EZ1 Advanced XL, which can process 14 samples in a run – is still on the market. However, it is too large to fit in a standard biosafety cabinet.

9.2 AIM

The overall aim of the evaluation was to find a new DNA-extraction robot that could replace the EZ1 Advanced for use in a BSL3 laboratory. A market survey was the first step. The most important criterion was the size of the robot since it was essential that it could be contained within a biosafety cabinet. Other criteria included which type of kits were available for the robot, delivery time for them, and costs for both for kits and the instrument. The second step was to compare the selected robots with the EZ1. The comparison was done by analysis of matrices important to each of the four agencies within FBD.

9.3 METHOD

The survey of DNA-extraction robots meeting our criteria (i.e. size, costs, delivery time etc.) was conducted by internet searches. Two different systems for automated isolation of nucleic acids of appropriate size – the magLEAD 6gC (Precision System Science Co. Ltd (PSS), Matsudo-city, Japan) and the InnuPure C16 (Analytik Jena AG, Jena, Germany) – were selected for evaluation; see figure 5 and table 8. The robots were made available by the respective company or retailer and transported to SVA in Uppsala for evaluation. First, a KI-discus-test was performed to ensure that the instruments would not interfere with the airflow in the biosafety cabinets in the BSL3 laboratory; see appendix 12.4. The magLEAD 6gC is the same size as the EZ1 Advanced system while the InnuPure C16 system is a little bit larger and wider; however, both systems passed the KI-discus-test.

[14] Projektrapport FBD 2009/3 - Utvärdering av extraktionsrobot 2009, Sara Frosth, Stina Bäckman, Linn Farhadi, Annelie Lundin Zumpe, Olga Stephansson och Joakim Ågren.

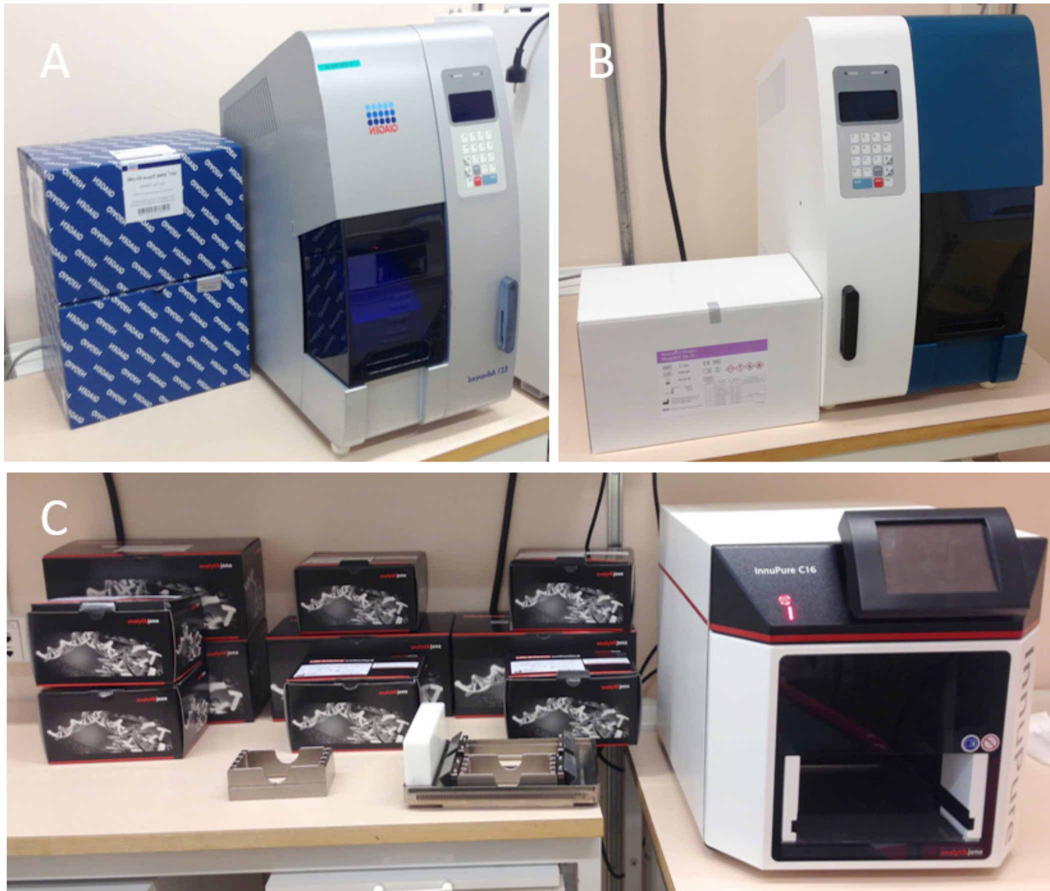

Figure 5. Panel A shows the EZ1 Advanced extraction robot from Qiagen, **panel B** the magLEAD 6gC from PSS, and **panel C** the InnuPure C16 from Analytic Jena.

Table 8. Characteristics of the extraction systems.

	EZ1 Advanced	magLEAD 6gC	InnuPure c16
Maximum samples per run	6	6	16
Time for extraction	15 minutes	25 minutes	Manual sample preparation (about 60 minutes) + 30-45 minutes
UV-treatment in robot	Yes	Yes	Can be purchased
All required reagents included in kit	Yes	Yes	No (some reagents for manual sample preparation are not included)
KI-discus-test for placement in biosafety cabinet (see appendix 12.4)	Passed	Passed	Passed
Reagents	Pre-portioned in strips	Pre-portioned in strips	Pre-portioned in strips or plates, foil is manually pierced

The two systems were evaluated by analysis of 20 matrices representative of the material usually analysed at the four different agencies in FBD. The EZ1 Advanced was also included in the evaluation for comparison; it uses EZ1 DNA Tissue Kits. A single kit, the MagDEA DX SV, was available for the magLEAD 6gC, while several different kits were available for the InnuPure C16. The kits for InnuPure C16 were matrix dependent. The choice of kit was decided after consultation with the manufacturer. The matrices were inoculated with the *F. tularensis* live vaccine strain (LVS) and *B. cereus* according to the *Protocol for evaluation of extraction robots*, see appendix 12.5, prior to DNA-extraction. The amount and quality of extracted DNA was measured on a Nanodrop or Picodrop spectrophotometer followed by real-time PCR analysis.

The targeted genes in the PCR-analysis were:

- *Bacillus rpoB*, which detects all species within the genus *Bacillus* and may detect background flora from the samples. These may be especially apparent after enrichment.
- *F. tularensis* ISFtu2, which is an insertion element used here to detect *F. tularensis* (LVS) that was inoculated in the samples. Since it is highly unlikely that *F. tularensis* is naturally present in the matrices included in this test, any detection of ISFtu2 in the negative extraction controls (NEC) would be indicative of cross-contamination in sample preparation or extraction process.
- Seal herpes virons (PhHV-1), which was included in the samples as an internal extraction control (IPC).

Since PCR master mixes vary in sensitivity to inhibitors derived from the matrices, three different mixes were used to evaluate the DNA extracts.

9.4 RESULTS OF THE EVALUATION

Baby food was a matrix difficult to handle for all of the robots; also spinach leaves and cream gave low concentrations of DNA. The results from the DNA measurements are presented in appendix 12.6. The magLEAD 6gC PSS instrument did handle some matrices, such as wheat flour and raspberries, better than the InnuPure C16. On the other hand, it had some problems in extracting DNA from tap water, as did the other robots. Figure 6 displays the number of real-time PCR runs with good results for each extraction robot and extraction kit. The complete real-time PCR results are shown in appendix 12.7.

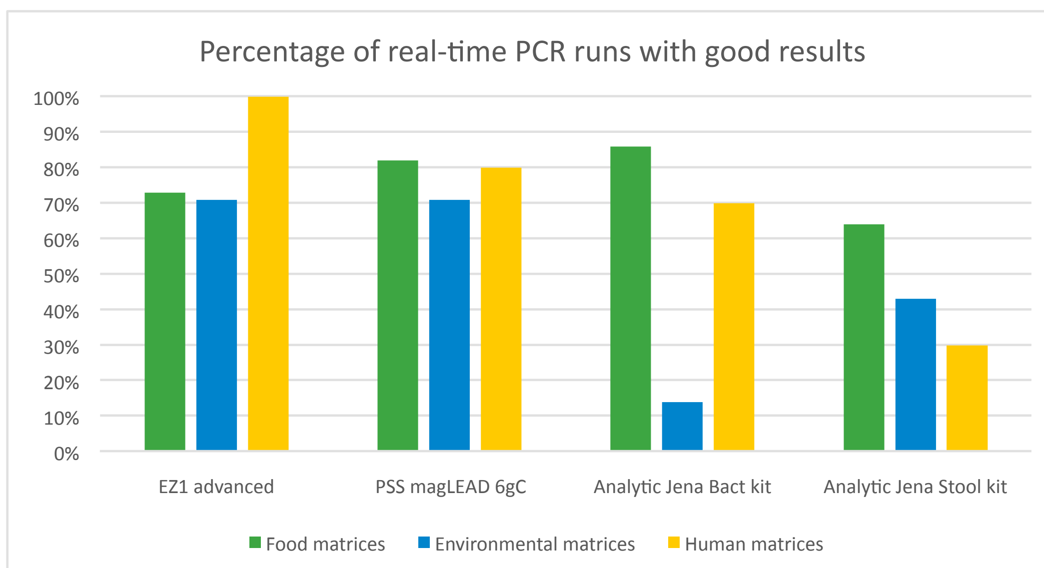

Figure 6. Percentage of real-time PCR runs with good results for different matrices. Food matrices included egg, raspberries, spinach, meat, baby food, cream, orange juice, chocolate, tap water and flour. Environmental matrices included water, soil, feed and mosquitoes. Clinical matrices included blood, spleen and swab samples.

9.5 DISCUSSION

The systems were compared for user friendliness and contamination risks. Certain pros and cons of extractions with the different systems are worth commenting below.

DNA extraction with the magLEAD 6gC instrument gave fairly good reproducibility of the results, comparable to that of the EZ1 instrument. On the other hand, the magLEAD 6gC was more time consuming and had a few practical obstacles that made it less user friendly to operate. Labels had a tendency to get stuck in the instrument and the software required many initial steps before the run could actually start. Furthermore, extracts from some matrices made with the magLEAD 6gC did not always support real-time PCR detection. For example, detection of *Bacillus* in raspberries and orange juice seemed to be inhibited.

The InnuPure C16 instrument, using the Stool-kit, was able to extract DNA from soil and egg that was detectable by real-time PCR-analysis. Extractions from these two matrices often contain high levels of substances that inhibit PCR-detection. However, a considerable disadvantage of the InnuPure C16 is that it is a more open system than the EZ1 Advanced and the magLEAD 6gC, which may increase the risk of contamination. The strips of reagents have to be pierced manually and there is no protective cover on the tips when loading them; this leads to a high risk of accidentally touching and contaminating them. Other disadvantages are that the instrument is space consuming, the kits do not contain all the reagents that are needed, and the delivery time of kits is long. Further, the extractions in the robot are preceded by a quite laborious sample preparation (see protocol, appendix 12.5), which prolongs the total time to produce DNA extracts. During the evaluation of the robots, the InnuPure C16 displayed "Device Error" in two runs. This required that everything loaded into the robot (samples, tips, tubes) had to be removed, a 15 min demo program had to be run, and the robot had to be reloaded with samples and consumables before the extraction could be performed. The overall impression of handling the instrument was that it is difficult and less user friendly than the instrument used today or the magLEAD 6gC.

As a result of this evaluation, the agencies of FBD invested in two PSS magLEAD 6gC robots. These will be placed at FOI and PHAS. For the BSL3 laboratory shared by NFA and SVA, the larger version of EZ1 Advanced instrument (14 samples capacity) was chosen.

10. FIELD EXERCISE: WATER SAMPLING AND ANALYSIS USING AN ULTRAFILTRATION METHOD

10.1 BACKGROUND

The ability to sample and analyse large volumes of water upon spread of disease or suspicion of microbiological contamination was one of the needs identified in the gap-analysis. Since the concentration of microbial contaminants in water tends to be low, analysis requires concentration of the microbes from a large volume of water. This can be done by various methods. The FBD agencies have previously implemented an ultrafiltration method that is used to concentrate microorganisms from large volumes of water[15]. More than 250 L drinking water can be filtered through the ultrafilter which entraps viruses, bacteria, and protozoa, due to its small pore size[16]. The ultrafilters can also be used to concentrate environmental water that contains more particles (e.g. sand, soil, algae). Here they can concentrate about 40-100 L, depending on the water turbidity.

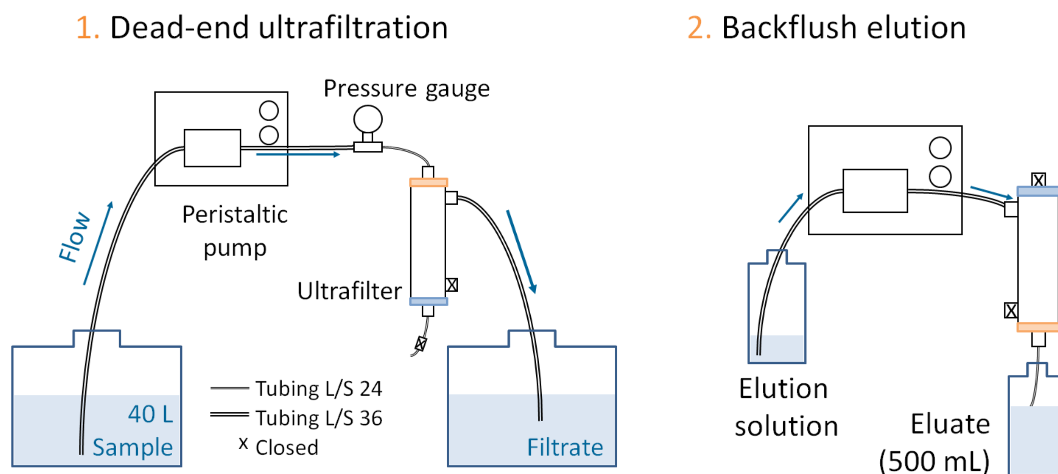

Figure 7. Water sampling by ultrafiltration. **Panel 1:** A large volume of water (40-250 L) is pumped through an ultrafilter by a peristaltic pump. All microorganisms, and particles larger than 30 kDa are contained in the filter and the filtrate is discarded. **Panel 2:** The backflush elution process flushes an elution solution through the filter to release microorganisms into an eluate, which can then be analysed by e.g., cultivation or PCR.

[15] The ultrafiltration method described in this report was kindly shared by Dr. Vincent Hill and his colleagues at the CDC.

[16] Smith, and Hill. (2009) Dead-end hollow-fiber ultrafiltration for recovery of diverse microbes from water, *Appl Environ Microbiol* 75, 5284-5289.

Staff was trained to conduct the ultrafiltration method upon setup at the FBD agencies in 2011 and 2012[17]. However, since this was several years ago, the need to revive the ability for water sampling and analysis of high-consequence agents was identified. Many of the staff members trained in the method no longer worked in this area, and hence their expertise was not available. Further, spread of high-consequence disease via water in Sweden has occurred in recent years. In anthrax outbreaks in cattle in Sweden in 2008 and 2011, water was identified as one possible route of transmission[18, 19]. Also *F. tularensis* can infect via water; in Sweden, cases of water-borne tularaemia from private wells were reported in 2012 and 2013 [20, 21].

Due to this, the FBD decided to perform education and training of staff to have national capability and redundancy in ultrafiltration for sampling and analysis of high-consequence agents in water. In addition to staff from NFA, SVA, PHAS, and FOI, the National CBRN Defence Centre (SkyddC) of the Swedish Armed Forces participated.

Before the exercise, all participating agencies ensured that 1-3 staff members became acquainted with the ultrafiltration method, i.e., read the protocols and performed a setup and test run of the sampling equipment.

10.2 AIM

The overall aim of the exercise was to ensure the national capability to retrieve and analyse a water sample possibly contaminated with highly pathogenic bacteria. This can be achieved, with a valuable redundancy, if the agencies within the FBD and SkyddC have staff with experience of using the ultrafiltration method in the field, transporting the sample to a BSL3 facility, and performing the relevant microbiological analyses.

Expected outcomes:

- Trained personnel that can prepare for sampling, take a large (>40 L) water sample by ultrafiltration, pack and transport samples, and perform the subsequent sample preparation and analysis.
- An improved checklist for packing and field sampling of water.
- Improved protocols for ultrafiltration of highly pathogenic bacterial agents.

[17] Lavander, Ågren, Karlsson, Hallin, and Stephansson. (2013) Detektion av högpatogena bakterier i vatten. Publ.nr. MSB576 ISBN: 978-91-7383-356-1.

[18] Lewerin, Elvander, Westermark , Hartzell, Norström, Ehrs, Knutsson, Englund, Andersson, Granberg, Bäckman, Wikström, Sandstedt. (2010) Anthrax outbreak in a Swedish beef cattle herd--1st case in 27 years: Case report. Acta Vet Scand. Feb 1;52:7.

[19] Larsson, Bergdahl (2011). Översvämning och mjältbrand. En analys av översvämningar och mjältbrand i Kvismaredalen. Länsstyrelsen Örebro län Publ. nr 2012:5.

[20] www.folkhalsomyndigheten.se The PHAS reports on statistics from surveillance of communicable diseases.

[21] Alm, Advani, Bråve, Wahab (2015). Draft genome sequence of strain R13-38 from a Francisella tularensis outbreak in Sweden. Genome announcements, 3(1), e01517-14.

10.3 SUMMARY OF THE EXERCISE

The exercise was performed during two days, with 4-5 persons taking part each day. The exercise directors brought the material and reagents needed to the laboratory at SVA. The participants were instructed to prepare for field sampling of 20 L of water using the ultrafiltration method. Filters were pre-treated with fetal calf serum and packed along with the other equipment before transportation to a nearby bay of the lake Mälaren.

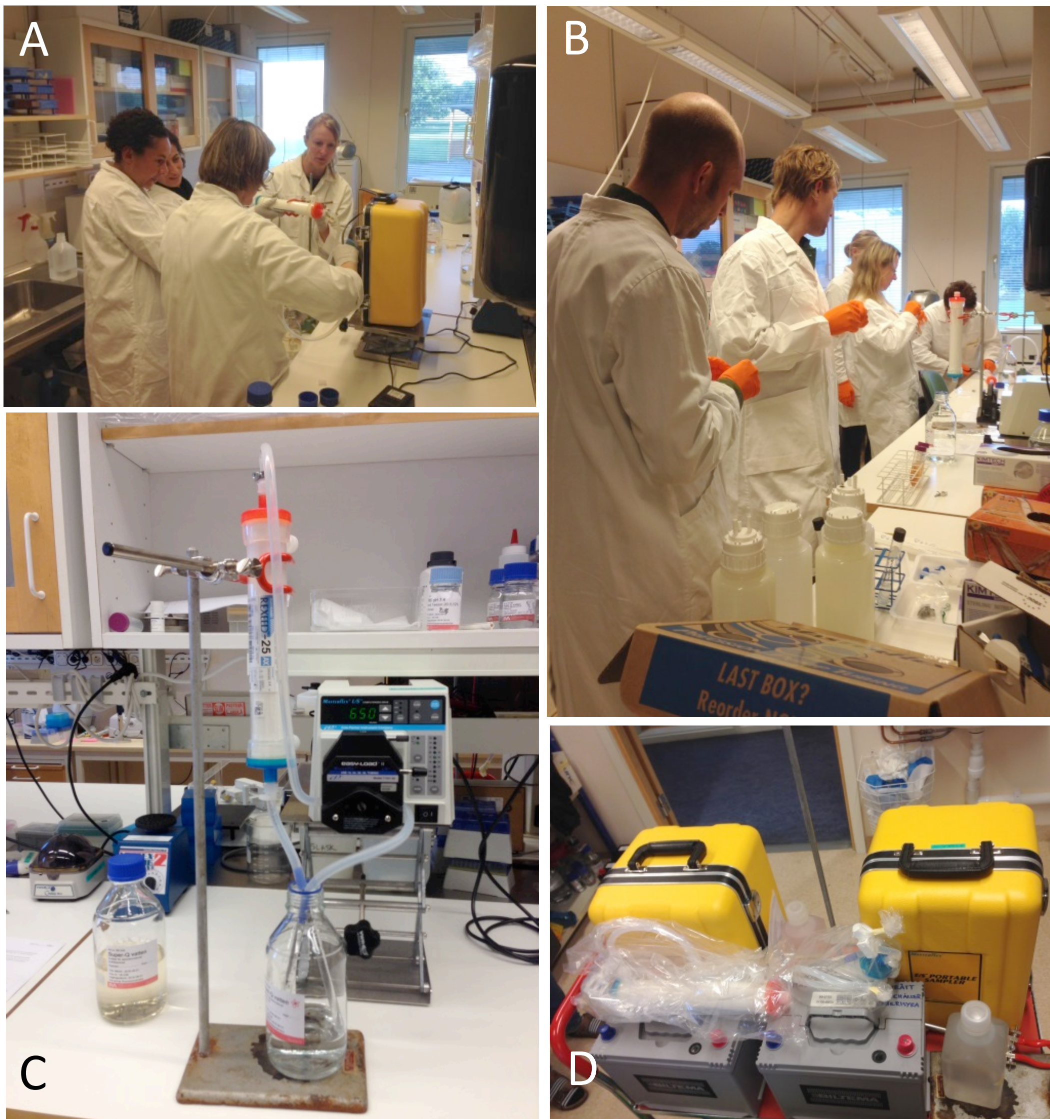

Figure 8. Panels A and B show the participants preparing material and packing equipment for field water sampling. In **panel C**, an ultrafilter is set up for pre-treatment with fetal calf serum. In **panel D**, the pre-treated filters are packed in plastic bags together with pumps, batteries and other equipment that will be brought to the sampling site.

At the sampling site, the exercise participants assembled the equipment and attached the pump to a battery for power. The water was taken from Mälaren and pumped through the filter to collect any particles larger than 30 kDa, including parasites, bacteria, and viruses. The staff learned to handle the battery, pump, filter, and tubing in a safe way to be able to use the method upon suspicion of high-consequence water contamination. When 20 L had been sampled, the setup was disassembled: tubing was detached, and the filters plugged and packed to be transported to the laboratory.

Figure 9. Panel A shows setup of sampling equipment at Mälaren. The portable pump (yellow) is powered by an RV battery (grey). The ultrafilter (with orange and blue ends) is mounted in a holder. A tarpaulin is used to demarcate the area where possibly contaminated water is handled. **Panels B** and **C** show the sampling. The water sample in the bucket is pumped into the filter and tubing leads the flow-through back into the lake. In **panel D**, the sampling has been completed. Most of the equipment has been repacked and all waste is put into a bag for contaminated material. Everything is brought back to the laboratory.

Back at the laboratory, the water samples were eluted from the filters in safety cabinets, mimicking how sample preparation would be performed in the BSL3 laboratory. The filter and the attached tubing can be quite cumbersome to handle within the cabinet. Further, since the tubing contains a concentrate of potentially contaminated water, it is important to avoid spills and splashes. It is therefore vital that the staff can train under safe circumstances before taking and preparing a real water sample. Another gain of the exercise was the possibility to work side by side with colleagues from other agencies and institutes and learn from each other both in the field and laboratory.

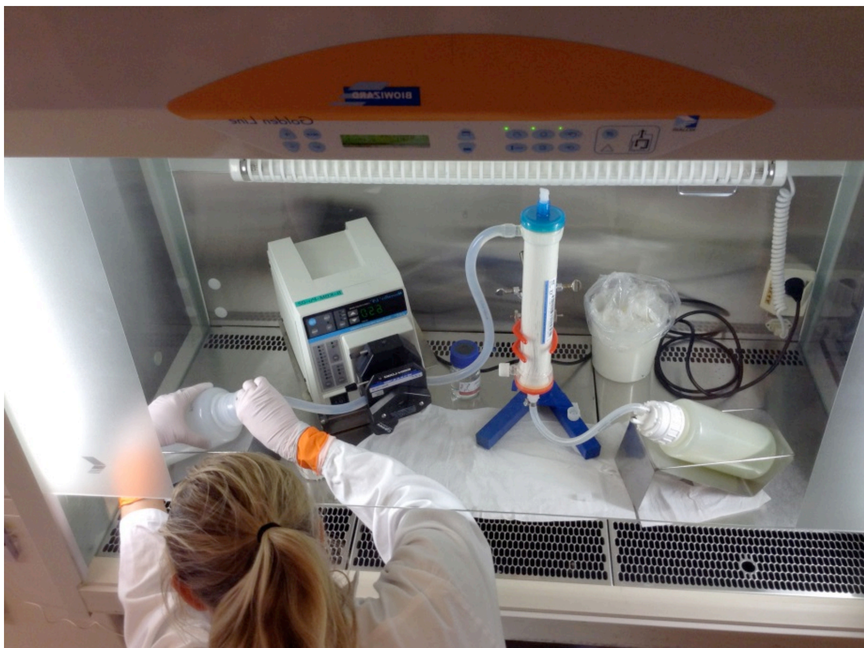

Figure 10. Setup of the ultrafilter and peristaltic pump in a safety cabinet, for elution of the sample from the filter. An elution solution is pumped from the left bottle through the filter, hence flushing the filter contents into the bottle to the right. In this case, the eluate is green from algae in the sampled water.

10.4 RESULTS

10.4.1 Outcome in brief

- A list of lessons learned and ideas resulting from the exercise.
- An improved packing checklist to perform a water sampling in the field.
- An improved protocol for ultrafiltration to sample and analyse water suspected of contamination with highly pathogenic bacteria.
- Foremost, staff could train for ultrafiltration sampling, take a large water sample, pack and transport the sample, and perform sample preparation for analysis of the eluate at a BSL3 laboratory.

10.4.2 Points drawn from the exercise and following discussions with the participants

- Clear and easy-to-follow protocols and checklists for packing are key.
- It is important to think before doing when working with complex methods such as ultrafiltration.
- Clear communication facilitates the workflow.
- It is crucial to be familiar with the risks of working with pathogenic microorganisms and in particular with water – where spills and splashes easily occur.
- It is valuable to be at least two persons, if possibly three, during field sampling to be able to work both safely and efficiently.
- Much was learned during the field sampling that is not obvious when learning the method under laboratory conditions, e.g., it is important to separate ”clean” and ”dirty” zones to minimise the risk of contamination. It was also learned that the bottom of the bucket fills up with sediment which can clog the filter; this sediment is therefore not used as part of the sample.
- To avoid spills of concentrated material from the filter, both in the field and when setting up the filter for elution in the laboratory, paper cloths soaked in disinfectant should be used to block filter openings. Failing to do this was the most common mistake during the exercise. Hence, this point must be clearly stated in the protocol. Currently it is only mentioned in the risk assessment for the method.
- During the exercise, consideration of risk and safety was sometimes neglected. In subsequent discussions, the participants stated that this was an effect from knowing that it was “just an exercise”. In general, they were all confident that the same neglect would not occur in a real event of water sampling and analysis. Hence there was a wish to make coming exercises more similar to the real event, e.g. by using full personal protection equipment.
- The environment was very well adapted for water sampling – a pier close to the water surface. For coming exercises it would be valuable to take the sample in a more problematic environment. This could be in a slope near a water-filled ditch, or in a muddy field by a pond where cattle graze.
- It is valuable if the exercise directors are only present as observers and do not offer any support in the activity. This prompts the participants to actively think, plan and discuss issues and questions.

10.5 DISCUSSION

In the evaluation, it was obvious that the participants found the exercise valuable. To maintain ability for rarely used and complex methods, training is necessary, preferably yearly. Another idea was to complement the practical training with instruction films for the methods. Making such films would require effort and resources but, once made, would be useful and efficient tools to uphold the knowledge of the method. As a suggestion, exercises and viewing of instructional film can be performed as biannual activities.

11. CONCLUDING REMARKS

It is a major challenge to maintain readiness for diagnostics and detection of rare microbial agents that are not routinely analysed in the laboratories. Staff, as well as instruments and methods, change over time and in order to maintain biopreparedness, it is crucial to continuously update, develop and harmonise methods. Staff training on a regular basis and established strategies for quality assurance are equally important.

One way to follow up on the capability for biopreparedness is gap-analysis. Gap-analysis is often a simple and fast way to inventory methods, techniques, and instrumentation. The result is a good base for planning and prioritising future work and activities. In this project, the four agencies conducted a joint gap-analysis. The result of the analysis gave a good indication of where effort was needed to maintain a good national capability.

As a result of the gap-analysis, this project developed and validated two new diagnostic methods (detection of species of *Yersinia* and *Brucella*); evaluated and tested two new instruments, i.e. a rapid test and an extraction robot; and planned and executed a field exercise in water sampling by ultrafiltration.

Nevertheless, gaps and needs shift over time. Therefore, it is of high importance to revise the inventory on a routine basis and to keep the priority list up to date. The expertise of the participating personnel can also influence the outcome of the gap-analysis. In this project, the first gap-analysis was performed in May 2014 and a priority list and working plan was elaborated. In January 2016, the gap-analysis was revised and other needs were identified. As a result, the priority list was revised and the objectives of the project changed direction.

For the future, the project group suggested that the agencies continue to keep the priority list up to date by routinely conducting a joint gap-analysis, for example once a year. The priority list would thereby be a dynamic document following the needs and requirements of the agencies.

11.1 RESULTS IN RELATION TO ORIGINAL OBJECTIVES.

Objective 1: To inventory/identify the participating agencies' need for better diagnostics of BSL3 agents.

Result: The inventory resulted in a priority list that constituted the base for further planning of the project in 2014 and 2015, (i.e., Objective 3 and a project for "Transfer knowledge and methods detecting *Francisella tularensis* to all agencies and with a focus on real-time PCR"). The inventory was repeated in 2016 resulting in a new priority list and changes of the project objectives (i.e., Objective "Transfer knowledge and methods detecting *Francisella tularensis* to all agencies and with a focus on real-time PCR" was omitted and Objectives 5 and 6 were added to the project).

Objective 2: To evaluate the IMASS rapid test developed by BBI Detection in the United Kingdom with respect to specificity and sensitivity for biological agents and the requirements of the Swedish Armed Forces and civilian actors such as the police.

Result: The project was performed in accordance with the objectives.

Objective 3: To develop and validate a real-time PCR diagnostic method for *Yersinia pestis, Y. pseudotuberculosis,* and *Y. enterocolitica*.

Result: A diagnostic method was developed and validated in accordance with the objective. The sensitivity as well as the efficiency of the PCR were very good, thus the new PCR fulfilled the criteria for implementation in FBD. During the validation, there was an additional objective added whereby the project would function as a pilot study for the new FBD implementation plan. During the process it became apparent that one of the agencies would implement the diagnostic assay.

Ojective 4: To develop and validate a real-time PCR diagnostic method for identifying all the biovars of *Brucella melitensis* and *suis*.

Result: The real-time PCR developed for *B. melitensis* showed very good specificity and sensitivity and was implemented at the three agencies' BSL3 laboratories. For *B. suis* an already published real-time PCR was implemented at two of the agency laboratories. The method was robust but detects only four *B. suis* biovars (biovars 1- 4) out of five.

Objective 5: To survey which DNA-extraction robots are currently available and suitable for a BSL3 facility. To evaluate how well the selected robots performed compared to the EZ1 Advanced in extraction and analysis of bacterial agents (*Bacillus* and *Francisella*) in relevant matrices.

Result: The project was performed in accordance with the objectives. As a result, two of the agencies purchased the PSS magLEAD 6gC instrument. For the BSL3 laboratory shared by NFA and SVA, the larger version of EZ1 Advanced instrument was chosen based on its capacity.

Objective 6: To train personnel in use of an ultrafiltration method for sampling and analysing large volumes of environmental water suspected to be contaminated with high-consequence agents.

Result: The project was performed in accordance with the objectives. Representatives from all agencies including personnel from the Swedish Armed Forces (SkyddC) participated in the exercises. In addition to training the personnel, the exercise resulted in a list of lessons learned and improvement of the protocol and the checklist for packing.

12 APPENDICES

12.1 EVALUATION OF THE IMASS

12.2 VALIDATION REPORT FOR DETECTION OF YERSINIA WITH REAL-TIME PCR

12.3 VALIDATION REPORTS FOR DETECTION OF BRUCELLA WITH REAL-TIME PCR

12.3.1 *Brucella melitensis*

12.3.2 *Brucella suis*

12.4 KI-DISCUS-TEST

12.4.1 EZ1 Advanced /magLEAD 6gC

12.4.2 InnuPure C16

12.5 PROTOCOL FOR EVALUATION OF EXTRACTION ROBOTS

12.6 DNA MEASUREMENTS FROM THE EXTRACTION ROBOT EVALUATION

12.6.1 Nanodrop

12.6.2 Picodrop

12.7 RESULTS FROM THE EXTRACTION ROBOT EVALUATION

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Projekt/Project	Sidnr/Page no
Utvärdering av IMASS-systemet	1 (14)

Projektnummer/Project no	Kund/Customer
E490502	BBI Detection
FoT-område	

Handläggare/Our reference	Datum/Date	Memo nummer/number
Emelie Näslund Salomonsson	22 January 2015	FOI Memo 5238

Evaluation of the IMASS-system

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The IMASS-system
Image is © BBI Detection Ltd

Sändlista/Distribution: Cate Smith, BBI Detection

1 Introduction

This project intends to evaluate the IMASS system (Integrated Multiplex Assay and Sampling System), developed by BBI Detection in the United Kingdom. The system consists of an integrated sponge and eight lateral flow immunoassays that can detect eight biological agents simultaneously: five bacteria and three toxins.

In this project we have focussed on three of the bacterial agents and two toxins. The tests have been carried out with regard to specificity and sensitivity. Studies were performed both on single test strips as well as on the complete system.

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2 Test description

2.1 Culturing and counting bacteria

Each bacterium tested was cultured at 37ºC on appropriate agar plates. Stock dilutions were prepared by transferring a small loop of bacteria into a tube containing phosphate buffered saline (PBS). Concentration (CFU/ml) was determined at OD_{600}=1 by plating tenfold dilution series and counting the number of colonies that appeared.

For testing the strain variants and near-neighbors, suspensions were diluted with BBI-buffer (produced by the manufacturer) to 10^7 CFU/ml (*Yersinia* 10^8 CFU/ml). The toxins were diluted in BBI-buffer to a concentration of 10 ng/ml. All tests were conducted in six-fold and scored by comparing the test strip with the “Rann” visual scoring card provided by BBI Detection. Based on the intensity of the lines on the “Rann” card we decided that a test needs to score at least 3 in order to be regarded as positive. The line defined as 2 on the “Rann” card is too weak.

2.2 Testing of the single test strips

1. Samples and dilutions were prepared in the buffer provided. A volume of 100 µl of the prepared sample dilution was pipetted in the cassette well of each test strip.
2. After applying, the samples should start to run in under a minute and be complete by approximately 15 minutes.
3. Each test was visually read after 2 and 15 minutes and scored by comparing the test strip with the “Rann” visual scoring card provided by BBI Detection.

2.3 Testing of the IMASS system (only LOD testing)

1. Samples and dilutions were prepared in the buffer provided. A volume of 100 µl of the prepared sample dilution was pipetted into a droplet on a petri dish.
2. The droplet was sampled with the integrated sponge and buffer was added. The device was placed vertically on a flat surface and the samples should start to run in under a minute and be complete by approximately 15 minutes.
3. Each test was visually read after 5 and 15 minutes and scored by comparing the test strip with the “Rann” visual scoring card provided by BBI Detection.

2.4 Tested biological agents

Biological agents
Bacillus anthracis (vegetative cells)
Francisella tularensis
Yersinia pestis
ricin
botulinum toxin A

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2.5 Strain variants and near-neighbors tested

Selection	Genus	Species	Strain	Strain no.	Comments
Targets	*Bacillus*	*anthracis*		BA 22100311	
	Bacillus	*anthracis*		4229	Missing pXO1
	Bacillus	*anthracis*		7702	Missing pXO2
	Bacillus	*anthracis*		BKT 47397	
	Bacillus	*anthracis*		BKT 76340	
Near-neighbors	*Bacillus*	*cereus*		CCUG 7414	
	Bacillus	*cereus*		ATCC 10987	
	Bacillus	*cereus*		F2095/98	
	Bacillus	*cereus*		NVH 0597/99	
	Bacillus	*cereus*		WSBC 10528	
Targets	*Francisella*	*tularensis*	*holarctica*	FSC200	
	Francisella	*tularensis*	*holarctica*	FSC021	B5 japonica
	Francisella	*tularensis*	*mediasiatica*	FSC148	
	Francisella	*tularensis*	*tularensis*	FSC237	A1 (SCHU S4)
	Francisella	*tularensis*	*tularensis*	FSC054	A2
Near-neighbors	*Francisella*	*novicida*		FSC159	
	Francisella	*novicida*		FSC040	
	Francisella	*hispaniensis*		FSC454	
	Francisella	*philomiragia*		FSC145	
	Francisella	*noatunensis*	*orientalis*	FSC770	
Targets	*Yersinia*	*pestis*		2028-04	
	Yersinia	*pestis*		10329-02	
	Yersinia	*pestis*		10029-02	
	Yersinia	*pestis*		570-04	
	Yersinia	*pestis*		144-03	
Near-neighbors	*Yersinia*	*enterocolitica*			
	Yersinia	*enterocolitica*			
	Yersinia	*bercovieri*			
	Yersinia	*kristensenii*			
	Yersinia	*pseudotuberculosis*			
Toxins	ricin				Pure
	agglutinin				
	botulinum toxin A				Complex
	botulinum toxin A				Pure

2.6 Limit of detection testing

The limit of detection (LOD) for each test strip was determined using the most responsive bacterial strain of each targeted species (results obtained from the strain variants testing results).
Following concentrations were used:

Agents	**Concentrations (strips) Bacteria CFU/ml Toxins ng/ml**				**Concentrations (IMASS) Bacteria CFU/ml Toxins ng/ml**			
F. tularensis	10^3	10^4	10^5	10^6	10^5	10^6	10^7	10^8
B. anthracis	10^4	10^5	10^6	10^7	10^4	10^5	10^6	10^7
Y. pestis	10^5	10^6	10^7	10^8	10^5	10^6	10^7	10^8
ricin	0,5	1	5	10	5	10	50	100
botulinum toxin A	1	5	10	50	-	10	50	100

Tests with strips were conducted in six-fold. Tests with the complete IMASS-system were conducted in three-fold.

3 Results

Positive results are highlighted in grey.

3.1 *Bacillus anthracis* (vegetative cells)

2 min Conc. 10^7 CFU/ml	**Strain**	**Test no.**					
		1	**2**	**3**	**4**	**5**	**6**
Strain variant	*Bacillus anthracis* 22 100311	0	0	0	0	0	0
	Bacillus anthracis 4229	0	0	0	0	0	0
	Bacillus anthracis 7702	0	0	0	0	0	0
	Bacillus anthracis 47397	0	0	0	0	0	0
	Bacillus anthracis 76340	0	0	0	0	0	0
Near-neighbors	*Bacillus cereus* CCUG 7414	0	0	0	0	0	0
	Bacillus cereus ATCC 10987	0	0	0	0	0	0
	Bacillus cereus F2095/98	0	0	0	0	0	0
	Bacillus cereus NVH 0597/99	0	0	0	0	0	0
	Bacillus cereus WSBC 10528	0	0	0	0	0	0

15 min Conc. 10^7 CFU/ml	**Strain**	**Test no.**					
		1	**2**	**3**	**4**	**5**	**6**
Strain variant	*Bacillus anthracis* 22 100311	3	4	7	8	10	9
	Bacillus anthracis 4229	0	0	0	0	0	0
	Bacillus anthracis 7702	0	0	0	0	0	0
	Bacillus anthracis 47397	2	4	4	4	3	3
	Bacillus anthracis 76340	5	5	5	6	6	5
Near-neighbors	*Bacillus cereus* CCUG 7414	0	0	0	0	0	0
	Bacillus cereus ATCC 10987	0	0	0	0	0	0
	Bacillus cereus F2095/98	0	0	0	0	0	0
	Bacillus cereus NVH 0597/99	0	0	0	0	0	0
	Bacillus cereus WSBC 10528	0	0	0	0	0	0

3.1.1 Limit of detection *Bacillus anthracis* (vegetative cells)

Strips Strain	Concentrations CFU/ml	Test no. 1	 2	 3	 4	 5	 6
2 min	10^4	0	0	0	0	0	0
Bacillus anthracis 76340	10^5	0	0	0	0	0	0
	10^6	0	0	0	0	0	0
	10^7	0	0	0	0	0	0
15 min	10^4	0	0	0	0	0	0
Bacillus anthracis 76340	10^5	0	0	0	0	0	0
	10^6	0	2	2	0	0	0
	10^7	4	5	4	5	5	5

IMASS Strain	Concentrations CFU/ml	Test no. 1	 2	 3
5 min	10^4	0	0	0
Bacillus anthracis 76340	10^5	0	0	0
	10^6	0	0	0
	10^7	0	0	0
15 min	10^4	0	0	0
Bacillus anthracis 76340	10^5	0	0	0
	10^6	0	0	0
	10^7	0	0	0

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3.2 *Francisella tularensis*

2 min Conc. 10^7 CFU/ml	**Strain**	**Test no.**					
		1	**2**	**3**	**4**	**5**	**6**
Strain variant	*Francisella tularensis holarctica* FSC200	4	6	6	6	6	6
	Francisella tularensis holarctica FSC021	6	6	6	6	7	7
	Francisella tularensis mediasiatica FSC148	4	6	6	6	6	6
	Francisella tularensis tularensis FSC237	4	3	3	4	4	4
	Francisella tularensis tularensis FSC054	4	4	4	4	3	3
Near-neighbors	*Francisella novicida* FSC159	0	0	0	0	0	0
	Francisella novicida FSC040	0	0	0	0	0	0
	Francisella hispaniensis FSC454	2	0	0	0	0	0
	Francisella philomiragia FSC145	0	0	0	0	0	0
	Francisella noatunensis orientalis FSC770	0	0	0	0	0	0

15 min Conc. 10^7 CFU/ml	**Strain**	**Test no.**					
		1	**2**	**3**	**4**	**5**	**6**
Strain variant	*Francisella tularensis holarctica* FSC200	10	10	10	10	10	10
	Francisella tularensis holarctica FSC021	10	10	10	10	10	10
	Francisella tularensis mediasiatica FSC148	10	10	10	10	10	10
	Francisella tularensis tularensis FSC237	10	10	10	10	10	10
	Francisella tularensis tularensis FSC054	10	10	10	10	10	10
Near-neighbors	*Francisella novicida* FSC159	0	0	0	0	0	0
	Francisella novicida FSC040	0	0	0	0	0	0
	Francisella hispaniensis FSC454	2	0	0	0	0	0
	Francisella philomiragia FSC145	0	0	0	0	0	0
	Francisella noatunensis orientalis FSC770	0	0	0	0	0	0

3.2.1 Limit of detection *Francisella tularensis*

Strips	**Concentrations CFU/ml**	**Test no.**					
Strain		**1**	**2**	**3**	**4**	**5**	**6**
2 min	10^3	0	0	0	0	0	0
Francisella tularensis mediasiatica FSC148	10^4	0	0	0	0	0	0
	10^5	0	0	0	0	0	0
	10^6	0	0	0	0	0	0
15 min	10^3	0	0	0	0	0	0
Francisella tularensis mediasiatica FSC148	10^4	0	0	0	0	0	0
	10^5	0	0	2	0	2	2
	10^6	5	5	5	5	5	5

IMASS	**Concentrations CFU/ml**	**Test no.**		
Strain		**1**	**2**	**3**
5 min	10^5	0	0	0
Francisella tularensis mediasiatica FSC148	10^6	3	0	3
	10^7	3	3	3
	10^8	6	6	6
15 min	10^5	2	0	0
Francisella tularensis mediasiatica FSC148	10^6	3	2	3
	10^7	5	6	6
	10^8	10	10	9

3.3 *Yersinia pestis*

2 min Conc. 10^8 CFU/ml	**Strain**	**Test no.** **1**	 **2**	 **3**	 **4**	 **5**	 **6**
Strain variant	*Yersinia pestis* 2028-04	4	4	4	2	4	4
	Yersinia pestis 10329-02	2	2	2	2	2	2
	Yersinia pestis 10029-02	2	2	2	0	2	2
	Yersinia pestis 570-04	2	4	2	2	2	2
	Yersinia pestis 144-03	2	2	2	2	2	2
Near-neighbors	*Yersinia enterocolitica*	0	0	0	0	0	0
	Yersinia enterocolitica	0	0	0	0	0	0
	Yersinia bercovieri	0	0	0	0	0	0
	Yersinia kristensenii	0	0	0	0	0	0
	Yersinia pseudotuberculosis	0	0	0	0	0	0

15 min Conc. 10^8 CFU/ml	**Strain**	**Test no.** **1**	 **2**	 **3**	 **4**	 **5**	 **6**
Strain variant	*Yersinia pestis* 2028-04	6	6	6	4	6	8
	Yersinia pestis 10329-02	4	4	4	4	4	4
	Yersinia pestis 10029-02	4	4	4	2	4	4
	Yersinia pestis 570-04	4	6	6	4	4	4
	Yersinia pestis 144-03	4	4	2	4	4	4
Near-neighbors	*Yersinia enterocolitica*	0	0	0	0	0	0
	Yersinia enterocolitica	0	0	0	0	0	0
	Yersinia bercovieri	0	0	0	0	0	0
	Yersinia kristensenii	0	0	0	0	0	0
	Yersinia pseudotuberculosis	0	0	0	0	0	0

3.3.1 Limit of detection *Yersinia pestis*

Strips Strain	Concentrations CFU/ml	Test no. 1	 2	 3	 4	 5	 6
2 min	10^5	0	0	0	0	0	0
Yersinia pestis 2028-04	10^6	0	0	0	0	0	0
	10^7	2	2	2	2	2	2
	10^8	4	4	4	4	4	4
15 min	10^5	0	0	0	0	0	0
Yersinia pestis 2028-04	10^6	0	0	0	0	0	0
	10^7	3	2	2	2	3	3
	10^8	6	6	6	5	6	8

IMASS Strain	Concentrations CFU/ml	Test no. 1	 2	 3
5 min	10^5	0	*	0
Yersinia pestis 2028-04	10^6	0	0	0
	10^7	0	0	*
	10^8	0	2	0
15 min	10^5	0	*	0
Yersinia pestis 2028-04	10^6	0	0	0
	10^7	0	0	0
	10^8	2	4	2

* The sample had difficulties reaching the strip.

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3.4 Ricin

2 min Conc. 10 ng/ml	Test no. 1	2	3	4	5	6
ricin	3	3	3	3	3	3
agglutinin	3	3	3	3	3	3

15 min Conc. 10 ng/ml	Test no. 1	2	3	4	5	6
ricin	7	7	7	7	6	7
agglutinin	8	9	8	9	8	8

3.4.1 Limit of detection ricin

Strips	Concentrations ng/ml	Test no. 1	2	3	4	5	6
2 min	0,5	0	0	0	0	0	0
ricin	1	0	0	0	0	0	0
	5	2	2	2	2	2	2
	10	3	3	3	3	3	3
15 min	0,5	2	2	2	2	2	2
ricin	1	3	3	3	3	3	3
	5	4	4	5	5	5	4
	10	7	7	7	7	6	7

IMASS	Concentrations CFU/ml	Test no. 1	2	3
5 min	5	0	0	0
ricin	10	0	0	0
	50	0	0	(2)
	100	2	2	*
15 min	5	(2)	(2)	(2)
ricin	10	2	2	2
	50	3	2	3
	100	3	4	4

* The sample had difficulties reaching the strip.

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3.5 Botulinum toxin A

2 min	**Test no.**					
Conc. 10 ng/ml	**1**	**2**	**3**	**4**	**5**	**6**
botulinum toxin A (complex)	0	0	0	0	0	0
botulinum toxin A (pure)	0	0	0	0	0	0

15 min	**Test no.**					
Conc. 10 ng/ml	**1**	**2**	**3**	**4**	**5**	**6**
botulinum toxin A (complex)	4	4	5	4	4	4
botulinum toxin A (pure)	4	4	3	4	4	4

3.5.1 Limit of detection botulinum toxin A

Strips	**Concentrations ng/ml**	**Test no.**					
		1	**2**	**3**	**4**	**5**	**6**
2 min	1	0	0	0	0	0	0
botulinum toxin A (complex)	5	0	0	0	0	0	0
	10	0	0	0	0	0	0
	50	3	3	2	2	2	2
15 min	1	1	1	0	0	0	0
botulinum toxin A (complex)	5	3	3	3	3	3	3
	10	4	4	3	4	4	4
	50	7	7	6	6	6	7

IMASS	**Concentrations CFU/ml**	**Test no.**		
		1	**2**	**3**
5 min	5	0	0	0
botulinum toxin A (complex)	10	0	0	0
	50	0	0	0
	100	(1)	0	0
15 min	5			
botulinum toxin A (complex)	10	0	0	0
	50	(2)	(1)	(1)
	100	3	2	2

4 Summary and conclusions

Target	Specificity	Cross-reactivity	LOD strips Bacteria CFU/ml Toxins ng/ml	LOD IMASS Bacteria CFU/ml Toxins ng/ml	Comments
B. anthracis (veg. cells)	Good		10^7	-**	Misses two modified strains lacking one virulence plasmid each
F. tularensis	Very good		10^6	10^6	
Y. pestis	Very good		10^7	-**	
ricin	(Good)*	Agglutinin	1	50	
botulinum toxin A	(Good)*		5	-**	

* Only two toxin samples were tested.

** The test were negative with the concentrations tested, se table in section 2.6.

The IMASS-system provides a simple and fast method in order to identify biological agents. Since the system analyses eight samples simultaneously it is a time-saving method. The IMASS-system is a very specific method and we cannot find any cross-reactivity except for ricin and agglutinin which was expected. Notably, the IMASS-system could not detect two modified *B. anthracis* strains, each lacking a virulence plasmid. Given that bacterial strains can alter their genetic content by adding or deleting DNA / plasmids, this is something that must be taken into account.

The limit of detection for ricin and botulinum toxin A is very good and the IMASS-system can detect botulinum toxin A both in a complex as well as in pure form. However, the system requires high bacterial concentrations and given that the sample needs to be distributed over eight stripes, there is also a requirement for larger sample volumes. Still, the purpose of the IMASS-system is to sample at "white powder" incidents where the concentration of a putative B-agent is likely to be relatively high.

FORUM FÖR
BEREDSKAPSDIAGNOSTIK

FBD

FBD 005-2
2013-12-18

1 PROTOCOL FOR VALIDATION OF A REAL-TIME PCR ASSAY FOR DETECTION OF BACTERIA

Name of the method
Probe-based real-time PCR for detection of *Yersinia pestis*, *pseudotuberculosis* and *enterocolitica*

Summary of results		
Specificity, inclusivity (%)	100 %	
Specificity, exclusivity (%)	100 %	
	PHAS	**SVA**
Efficiency (%) and CI	102 % +/- 0.01190	105 % +/- 0.00072
LOD (GE/reaction)	6.25	6.25
Precision, repeatability (CV %)	1.30	2.32
Precision, reproducibility (CV %)	-	-
Accuracy:	-	-
Robustness:	Evaluated	Evaluated

Date	**Performed by**	**Approved by**
2015-12	*Johanna Thelaus, FOI* *Maria Wijkander, PHAS* *Joann Börjesson, SVA* *Sara Frosth, SVA* *Paula Ågren, NFA*	*Emelie Näslund Salomonsson, FOI*

1.1 BACKGROUND AND AIM

Aim
To be able to detect *Yersinia pestis*, *pseudotuberculosis* and *enterocolitica* by real time-PCR.

Background
Previously, there has not been a PCR method that can detect all three *Yersinia* species simultaneously.

Parameters to be validated

☒ Specificity
☒ LOD
☒ Efficiency
☒ Precision: repeatability
☐ Precision: reproducibility
☐ Accuracy
☒ Robustness

Associated protocols

FBD 004 – Protocol for preparation of primers and probes
Used ☐ Yes ☒ No
We enlisted the help of Erik Alm at PHAS which has developed a program, SmiPrimer, to develop and optimize primers and probes.

FBD 007 – Protocol for preparation of reference and control material (DNA)
Used ☐ Yes ☒ No
We used existing DNA (phHV-1, phocine herpes virus) as an internal positive control which previously has been developed by PHAS.

Protocols used for validation:

Name	FBD or other	Agency (if other)
FBD 005-2	FBD	

1.2 MATERIALS AND METHODS

Y. pestis (PHAS)

Primers and probes

Name of oligos	Sequence	Manufacturer	Batch number
Ye_gen_F	CGGTAYCTGTTGGGCTTTCCT	Life Technologies	5903708-40-4.1
Ye_gen_R	CATTAGCCGATTTCAATTTATGCTC	Life Technologies	5903708-50-5.1
Yp_Ypt_gen_F	TGTACCCGTTGGGCTTTCCT	Life Technologies	5903708-20-2.1
Yp_Ypt_gen_R	TGGCCGATTTCAGTTTATGCTC	Life Technologies	5903709-30-3.1
Y_gen_FAM-MGB	CTGATGTGTTGTTGAACCG	Life Technologies	5903708-10-1.1

Name of internal controls	Sequence	Manufacturer	Batch number
IAC_F	GGGCGAATCACAGATTGAATC	Life Technologies	5903708-60-6.1
IAC_R	GCGGTTCCAAACGTACCAA	Life Technologies	5903708-70-7.1
IAC_VIC-TAMRA	TTTTATGTGTCCGCCACCATCTGGATC	Life Technologies	5903708-80-8.1

Control DNA

Strain	Batch/reference	Extraction method	Measurement	Concentration
KIM5	FOI	EZ1 DNA extraction robot (Qiagen) EZ1 DNA tissue kit (Qiagen)	Qubit	8.28 ng/uL

Positive DNA control and internal control

Control	Description	Batch number or date for manufacturing	Thawed (date)
Positive	*Y.pestis* NCTC 2028	2011-05-11	151110
IAC	phHV-1	2015-02-26	151110/151116
NC	Water	Lot: 1660064	

Mastermix

Regents	Name	Manufacturer	Batch number	Concentra-tion (stock)	Volume (µl)
H_2O	DNAse & RNAs-free	Life technologies	Lot:1660064 REF: 10977-035		2.75
Mastermix*	PerfeCTa® qPCR ToughMix (VWR)	Quanta BioSciences	19846	2x	12.5
Primer 1:	Ye_gen_F	Life Technologies	5903708-40-4.1	20 µM	0.625
Primer 2:	Ye_gen_R	Life Technologies	5903708-50-5.1	20 µM	0.625
Primer 3:	Yp_Ypt_gen_F	Life Technologies	5903708-20-2.1	20 µM	0.625
Primer 4:	Yp_Ypt_gen_R	Life Technologies	5903709-30-3.1	20 µM	0.625
Probe 1:	Y_gen_FAM_MGB	Life Technologies	5903708-10-1.1	5 µM	0.25
Control primer 1(IAC):	IAC_F	Life Technologies	5903708-60-6.1	20 µM	0.625

Control primer 2(IAC):	IAC_R	Life Technologies	5903708-70-7.1	20 µM	0.625
Probe control (IAC):	IAC_VIC-TAMRA	Life Technologies	5903708-80-8.1	5 µM	0.25
Internal control template (IAC):	phHV-1	SMI		(10^{-6}) Ct:30	0.5
Template					5
Total					**25**

Oligos (stock solution)

Stock solution	Manufacturing date	Thawed (times)
Ye_gen_F	151110	1
Ye_gen_R	151110	1
Yp_Ypt_gen_F	151110	1
Yp_Ypt_gen_R	151110	1
Y_gen_FAM_MGB	151110	1

PCR-program

Program	Temp (°C)	Time (min)	
Initial denaturation	95	3	
Cycling			**Number of cycles**
Denaturation of DNA	95	0:03	
Hybridisation and elongation	60	0:30	45

Instruments

Instrument	Model	Manufacturer	Service of instrument
PCR machine	SteponePlus	Applied biosystems	2015-01-29
PCR machine	SteponePlus	Applied biosystems	2015-01-29
Extraction robot	EZ1	Qiagen	2015-05-25

Y. pseudotuberculosis (FOI)

Primers and probes

Name of oligos	Sequence	Manufacturer	Batch number
Ye_gen_F	CGGTAYCTGTTGGGCTTTCCT	Eurofins MWG operon	019691808
Ye_gen_R	CATTAGCCGATTTCAATTTATGCTC	Eurofins MWG operon	019691810
Yp_Ypt_gen_F	TGTACCCGTTGGGCTTTCCT	Eurofins MWG operon	019691807
Yp_Ypt_gen_R	TGGCCGATTTCAGTTTATGCTC	Eurofins MWG operon	019691809
Y_gen_FAM_MGB	CTGATGTGTTGTTGAACCG	Life Technologies	104136-C5

Name of internal controls	Sequence	Manufacturer	Batch number
IPC_F	GGGCGAATCACAGATTGAATC	Eurofins MWG operon	020732517
IPC_R	GCGGTTCCAAACGTACCAA	Eurofins MWG operon	020732518
IPC_VIC_TAMRA	TTTTTATGTGTCCGCCACCATCTGGATC	Eurofins MWG operon	104969-C8

Positive DNA control and internal control

Control	Description	Batchnumber or date for manufacturing	Thawed (date)
Positive control	-		
IAC	phHV-1	PHAS	
NC	Water		

Mastermix

Regents	Name	Manufacturer	Batch number	Concen-tration (stock)	Volume (µl)
H_2O			-		2.25
Mastermix*	PerfeCTa® qPCR ToughMix #733-2090 (VWR)	Quanta BioSciences	22137	2x	12.5
Primer 1:	Ye_gen_F	Eurofins MWG operon	019691808	20 µM	0.625
Primer 2:	Ye_gen_R	Eurofins MWG operon	019691810	20 µM	0.625
Primer 3:	Yp_Ypt_gen_F	Eurofins MWG operon	019691807	20 µM	0.625
Primer 4:	Yp_Ypt_gen_R	Eurofins MWG operon	019691809	20 µM	0.625
Prob 1:	Y_gen_FAM_MGB	Life Technologies	104136-C5	5 µM	0.5
Control primer 1(IAC):	IPC_F	Eurofins MWG operon	020732517	20 µM	0.625
Control primer 2(IAC):	IPC_R	Eurofins MWG operon	020732518	20 µM	0.625
Probe control (IAC):	IPC_VIC_TAMRA	Eurofins MWG operon	104969-C8	5 µM	0.5
Internal control template (IAC):	phHV-1	-	-	(10^{-6}) Ct:30-31	0.5
Template	Ypt DNA				5
Total					**25**

Oligos (stock solution)

Stock solution	Manufacturing date	Thawed (times)
Ye_gen_F	150420	2
Ye_gen_R	150420	2
Yp_Ypt_gen_F	150420	2
Yp_Ypt_gen_R	150420	2
Y_gen_FAM_MGB	150420	2

PCR-program

Program	Temp (°C)	Time (min)	
Initial denaturation	95	3	
Cycling			**Number of cycles**
Denaturation of DNA	95	0:03	
Hybridisation and elongation	60	0:30	45

Instruments

Instrument	Model	Manufacturer	Service
PCR machine	CFX-96	Biorad	2015-02-10
Extraction robot	EZ1	Qiagen	2015-05-15

Y. enterocolitica and exclusivity panel (SVA and NFA)

Primers and probes

Name of oligos	Sequence	Manufacturer	Batch number
Ye F	CGGTAYCTGTTGGGCTTTCCT	Applied biosystems	2360771
Ye R	CATTAGCCGATTTCAATTTATGCTC	Applied biosystems	2360772
Yp Ypt F	TGTACCCGTTGGGCTTTCCT	Applied biosystems	2360769
Yp Ypt R	TGGCCGATTTCAGTTTATGCTC	Applied biosystems	2360770
Yp Ye Ypt prob FAM	CTGATGTGTTGTTGAACCG	Applied biosystems	2360767

Name of internal controls	Sequence	Manufacturer	Batch number
IAC F	GGGCGAATCACAGATTGAATC	Applied biosystems	2360773
IAC R	GCGGTTCCAAACGTACCAA	Applied biosystems	2360774
IAC prob VIC	TTTTTATGTGTCCGCCACCATCTGGATC	Applied biosystems	2360768

Control DNA

	Strain	Batch/reference	Extraction method	Measurement	Concentration
SVA	SLV 408		EZ1 DNA extraction robot (Qiagen) EZ1 DNA tissue kit (Qiagen)	Qubit	40.5 ng/uL
SLV	SLV 408		EZ1 DNA extraction robot (Qiagen) EZ1 DNA tissue kit (Qiagen)	Qubit	2 ng/uL

Positive DNA control and internal control

Control	Description	Batch number or date for manufacturing	Thawed (date)
Positive control	SLV 408		
IAC	phHV-1	PHAS	
NC	Water		

Mastermix

Regents	Name	Manufacturer	Batch number	Concen-tration (stock)	Volume (µl)
H_2O	DNAse & RNAs-free	Sigma	RNBD2922		2.75
Mastermix* - SVA	PerfeCTa® qPCR ToughMix (VWR)	Quanta BioSciences	Lot no 22004	2x	12.5
Mastermix* - SLV	PerfeCTa® qPCR ToughMix (VWR)	Quanta BioSciences	16724 Open 131113	2x	12.5
Primer 1:	Ye_gen_F	Applied biosystems	2360767	20 µM	0.625
Primer 2:	Ye_gen_R	Applied biosystems	2360769	20 µM	0.625
Primer 3:	Yp_Ypt_gen_F	Applied biosystems	2360770	20 µM	0.625
Primer 4:	Yp_Ypt_gen_R	Applied biosystems	2360771	20 µM	0.625
Prob 1:	Y_gen_FAM_MGB	Applied biosystems	2360772	5 µM	0.25
Control primer 1(IAC):	IAC_F	Applied biosystems	2360773	20 µM	0.625
Control primer 2(IAC):	IAC_R	Applied biosystems	2360774	20 µM	0.625
Probe control (IAC):	IAC_VIC-TAMRA	Applied biosystems	2360768	5 µM	0.25
Internal control template (IAC):	phHV-1	SMI		(10^{-6}) Ct:30	0.5
Template					5
Total					**25**

Oligos (stock solution)

Stock solution	Manufacturing date	Thawed (times)
Ye F	151110	2
Ye R	151110	2
Yp Ypt F	151110	2
Yp Ypt R	151110	2
Y_gen_FAM_MGB	151110	2

PCR-program

Program	Temp (°C)	Time (min)	
Initial denaturation	95	3	
Cycling			**Number of cycles**
Denaturation of DNA	95	0:03	
Hybridisation and elongation	60	0:30	45

Instrument

	Instrument	Model	Manufacturer	Service
SVA	PCR machine Diagnostiken	7500 Fast	Applied biosystems	2015-07-02
SVA	PCR machine FoU	7500 Fast	Applied biosystems	2014-05-07 (2015-09-08)*
SVA	Extraction robot	EZ1	Qiagen	2015-08-20
NFA	PCR machine	7500	Applied biosystems	2013-03-26
NFA	Extraction robot	EZ1	Qiagen	2015-01-26

* The equipment underwent service under contract by Life Technologies 2014-05-07. The last service was carried out by staff at the SVA 2015-09-08.

1.3 PREDEFINED SPECIFICATIONS

Recommended performance requirements

Specificity requirements (inclusivity and exclusivity):
100 %

Minimum acceptable value for Efficiency, E (%):
Between 90 and 110 %

Limit of detection, LOD (GE/µl):
Currently there are no defined demands on the LOD.

1.4 BENCHMARKS

Controls

Control	Cq (average)	Standard deviation
Negative PCR control (NC)	-	-
Internal amplification control (IAC)	29-31	-
Positive PCR control	-	-

Recommended values for dilution series

Dilution serie	
LOD (GE/reaction and Cq)	-
Dilutions (GE/reaction)	10^6 – 0.039

1.5 RESULTS AND CALCULATIONS

1.5.1 Specificity

Inclusivity

Y. pestis (PHAS)

No	Agent	Strain info	PCR 1 Cq	PCR 2 Cq	PCR 3 Cq	IAC Cq	Deviations
1	*Y.pestis*	144-03	19.24	19.18		41.52	
2	*Y.pestis*	570-04	19.36	19.44		41.64	
3	*Y.pestis*	2028-04	20.94	20.94		40.44	
4	*Y.pestis*	2868-03	21.25	22.1		40.73	
5	*Y.pestis*	8775-03	19.01	19.12		41.66	
6	*Y.pestis*	8779-04	20.25	20.28		40.55	
7	*Y.pestis*	10029-03	21.06	21.22		41.95	
8	*Y.pestis*	10030-01	21.33	21.56		42.81	
9	*Y.pestis*	10329-02	21.63	21.74		38.95	
10	*Y.pestis*	10330-03	18.99	19.24		41.21	
11	*Y.pestis*	Vietnam SBL 781010	35.13	34.75		34.44	
12	PC		32.13	32.02	32.33	32.75	
13	NC		N/A	N/A	N/A	N/A	

Y. pseudotuberculosis (FOI)

No	Agent	Strain info	PCR 1 Cq	PCR 2 Cq	PCR 3 Cq	IAC Cq	Deviations
1	*Y. pt.*	NCTC 10275	21.85	21.82	28.31	28.15	
2	*Y. pt.* YP III		22.56	22.55	27.75	27.84	
3	*Y. pt.* YP III		21.38	21.29	28.45	27.65	
4	*Y. pt.* YP III		22.50	22.86	27.61	27.83	

5	*Y. pt.* O1a	H 141/84	21.63	21.49	27.77	27.58	
6	*Y. pt.* O2a	H457/86	22.73	22.53	27.72	27.30	
7	*Y. pt.* O2b	H 143/84	21.08	21.25	27.33	27.92	
8	*Y. pt.* O2c	H 460/86	22.56	22.58	27.81	28.05	
9	*Y. pt.* O3	H 146/86	21.87	21.88	27.40	26.97	
10	*Y. pt.* O4a	H 452/86	22.63	23.06	26.85	27.15	
11	*Y. pt.* O4b	H 715/86	22.11	22.05	27.34	27.19	
12	*Y. pt.* O5b	H 450/86	21.96	22.08	27.11	27.38	
13	*Y. pt.* O8	H 448/86	22.55	22.48	27.35	27.15	
14	*Y. pt.* O10	H 16/92	21.64	21.66	27.48	27.92	
15	*Y. pt.* O14	H 918/92	21.71	21.63	27.25	27.39	
16	*Y. pt.* O15	H 6516/94	21.87	21.93	27.17	27.10	
17	*Y. pt.* O16	H 1180/95	21.60	21.55	27.32	27.46	
18	*Y. pt.* O1b	H 706/86	22.27	22.34	27.68	27.72	
19	*Y. pt.* O6	H 720/86	22.19	22.20	27.86	27.86	
20	*Y. pt.* O7	H 455/86	22.21	22.18	27.83	27.57	
21	NC		-	-	28.14	28.09	

Y. enterocolitica (SVA)

No	Agent	Strain info	PCR 1 Cq	PCR 2 Cq	PCR 3 Cq	IAC Cq	Deviations
1	*Y. enterocolitica* 1	Swine/feces	18.6	32.4	18.6	31.9	
2	*Y. enterocolitica* 2	Swine/feces	18.5	31.9	18.5	32.9	
3	*Y. enterocolitica* 3	Swine/feces	18.6	33.2	18.5	33.2	
4	*Y. enterocolitica* 4	Swine/feces	18.4	32.6	18.4	33	
5	*Y. enterocolitica* 5	Swine/feces	18	32.7	18	33.4	
6	*Y. enterocolitica* 6	Swine/feces	18.2	32.5	18.2	32.8	
7	*Y. enterocolitica* 7	Swine/feces	18.3	32.8	18.2	32.8	
8	*Y. enterocolitica* 8	Swine/feces	17.6	31.9	17.7	32	
9	*Y. enterocolitica* 9	Swine/feces	17.9	32	18.9	31.8	
10	*Y. enterocolitica* 10	Swine/feces	17.3	32.8	17.3	32.7	
11	*Y. enterocolitica* 11	Swine/feces	17.6	33.5	17.6	33.3	
12	*Y. enterocolitica* 12	Swine/feces	18.2	32.8	18.1	33.0	
13	*Y. enterocolitica* 13	Swine/feces	18.3	33.2	18.3	33.3	
14	*Y. enterocolitica* 14	Swine/feces	18.4	33.5	18.4	33.1	
15	*Y. enterocolitica* 15	Swine/feces	17.9	32.8	17.9	32.8	
16	*Y. enterocolitica* 16	Swine/feces	18.2	32.2	18.2	32.2	
17	*Y. enterocolitica* 17	Swine/feces	17.6	31.4	17.7	31.6	
18	*Y. enterocolitica* 18	Swine/feces	17.7	31.7	17.7	31.7	
19	PC		18.8	32.1	18.9	31.5	
20	NC		-	30.1			
21	NC		-	30			
22	NC		-	30.2			
23	*Y. enterocolitica* 19	Swine/feces	17.8	34.1	17.8	33.2	
24	*Y. enterocolitica* 20	Swine/feces	17.9	34.3	17.8	35.2	
25	*Y. enterocolitica* 21	Swine/feces	17.8	34.3	17.7	35.9	
26	*Y. enterocolitica* 22	Swine/feces	18.3	33.8	18.3	36.4	

27	*Y. enterocolitica* 23	Swine/feces	17.9	35.2	17.9	35.3	
28	*Y. enterocolitica* 24	Swine/feces	17.9	34.8	17.8	36.9	
29	*Y. enterocolitica* 25	Swine/feces	17.9	34.7	17.9	34.7	
30	*Y. enterocolitica* 26	Swine/feces	18.2	33.5	18.2	34	
31	*Y. enterocolitica* 27	Swine/feces	18.2	31.3	18.1	32.8	
32	*Y. enterocolitica* 28	Swine/feces	17.9	33.8	18	34.5	
33	*Y. enterocolitica* 29	Swine/feces	18	34.6	18	34.8	
34	*Y. enterocolitica* 30	Swine/feces	17.9	36.8	17.9	35.6	
35	*Y. enterocolitica* 31	Swine/feces	17.8	36.8	17.9	34.9	
36	*Y. enterocolitica* 32	Swine/feces	17.7	36.8	17.9	35.5	
37	*Y. enterocolitica* 33	Swine/feces	18.1	34	18.2	33.3	
38	*Y. enterocolitica* 34	Swine/feces	18	32.8	18.1	32.7	
39	*Y. enterocolitica* 35	Swine/feces	17.9	32.1	17.9	31.9	
40	*Y. enterocolitica* 36	Swine/feces	18.5	32.2	18.6	32.1	
41	*Y. enterocolitica* 37	Swine/feces	18.1	34.5	18.1	32.8	
42	*Y. enterocolitica* 38	Swine/feces	17.8	34.5	17.9	33.6	
43	*Y. enterocolitica* 39	Swine/feces	17.7	34.3	17.7	33.9	
44	*Y. enterocolitica* 40	Swine/feces	18.1	33.7	18.1	33.1	
45	*Y. enterocolitica* 41	Swine/feces	18.2	32.9	18.2	32.4	
46	*Y. enterocolitica* 42	Swine/feces	17.8	32	17.7	32	
47	*Y. enterocolitica* 43	Swine/feces	18.2	32.3	18.2	31.9	
48	*Y. enterocolitica* 44	Swine/feces	18	31.9	17.9	33.4	
49	*Y. enterocolitica* 45	Swine/feces	17.9	32.9	17.7	34.1	
50	*Y. enterocolitica* 46	Swine/feces	17.5	33.9	17.5	35.3	
51	*Y. enterocolitica* 47	Swine/feces	17.8	34	17.9	34.8	
52	*Y. enterocolitica* 48	Swine/feces	17.9	32.7	17.9	33.4	
53	*Y. enterocolitica* 49	Swine/feces	18.5	31.6	18.4	32.2	
54	*Y. enterocolitica* 50	Swine/feces	18.4	31.6	18.4	32.2	
55	*Y. enterocolitica* 51	Beef/feces QA	17.9	34	17.9	35	
56	*Y. enterocolitica* 52	Golden-headed lion tamarin	18.5	34.6	18.5	36.1	
57	*Y. enterocolitica* 53	Dog/feces	18.4	35.8	18.4	36.1	
58	*Y. enterocolitica* 54	Dog/feces	18.1	35.6	18.1	35.7	
59	*Y. enterocolitica* 55	Dog/feces QA	18.3	32.8	18.2	33.4	
60	*Y. enterocolitica* 56		18.9	32.2	18.9	31.9	
61	PC		18.8	34	19	35	
62	NC		-	29.9			
63	NC		-	29.8			
64	NC		-	29.7			

Results, inclusivity

Identified		87
Total number		87
Inclusivity (%)		100 %

Exclusivity

(NFA)

No	Agent	Strain info	Target 1 Cq:	IAC Cq	Deviations
1	*Campylobacter coli*	SLV-271	-	31.055965	
2	*Listeria ivanovii*	SLV-348	-	31.067558	
3	*Salmonella typhimurium*	SLV-248	-	31.055626	
4	*Proteus mirabilis*	SLV-374	-	31.183075	
5	*Escherichia coli*	U226	-	31.054771	
6	*E. coli*	B266	-	31.12099	
7	*E. coli*	L278	-	31.098835	
8	*E. coli*	UM245	-	31.200323	
9	*E. coli*	S262	-	31.449163	
10	*P. aeruginosa*	SLV-395	-	31.307686	
11	*C. jejuni*	SLV-542	-	31.03163	
12	*L. monocytogones*	SLV-513	-	30.996029	
13	*S. dublin*	SLV-424	-	31.269806	
14	*Enterobacter cloceae*	SLV-011	-	31.157705	
15	*Staphylococcus aureus*	SLV-438	-	31.32321	
16	*Enterococcus duran*	SLV-078	-	31.222889	
17	*P. aeruginosa*	SLV-453	-	31.036423	
18	*Klebsiella pneumonaie*	SLV-186	-	31.186707	
19	*Vibrio cholerae*	CCUG 4070	-	31.245895	
20	*Vibrio parahaemolyticus*	CCUG 4224	-	31.344524	
21	*Vibrio vulnificus*	CCUG 16397	-	31.154099	
22	*Bacillus cereus*	B. cereus	-	31.18996	
23	*Y. pestis*	KIM	25.881695	30.844173	
24	*Francisella tularensis*	T8	-	31.528067	
25	*B. anthracis*	7702	-	31.221523	
26	*B. anthracis*	4429	-	31.158506	
27	*E. coli O157*	SLV-479	-	31.086468	
28	*E. coli O157*	EDL933	-	31.291683	
29	*E. coli O113:H21*	98NK2	-	31.101894	
30	*E. coli O157:H-*	493/89	-	30.981651	
31	*E. coli*	XL- 1 blue	-	31.205332	
32	*E. coli O26:H11*	H2954/06	-	31.234478	
33	*E. coli EIEC*	121	-	31.144827	
34	*Shigella dysenterieae*	15/08	-	31.06787	
35	*S. flexneri*	100/08	-	31.03836	
36	*S. boydii*	33/08	-	31.207329	

37	*S. sonnei*	99/08	-	31.10698	
38	*Fusarium graminearum*	F. g	-	31.011124	
39	*F. culmorum*	F. c	-	31.260592	
40	*Y. fredriksenii*	99828	-	31.554169	
41	*Y. fredriksenii*	98731	-	31.574928	
42	*Y. intermedia*	Y. int	-	31.80209	
43	*Y. intermedia*	2202	-	31.616304	
44	*Y. intermedia*	SLV-472	-	31.351068	
45	*Y. kristensenii*	2147	-	31.497843	
46	*Y. kristensenii*	SLV-286	-	31.69662	
47	*Y. ruckeri*	941027	-	31.52135	
48	*Y. bercovieri*	Y36/15	-	31.426895	
49	*Y. bercovieri*	Y42/15	-	31.350975	
50	*Y. bercovieri*	Y45/15	-	31.528976	
51	*Y. mollaretii*	Y6/10	-	31.706936	
52	*Y. mollaretii*	Y25/12	-	31.621752	
53	*Y. mollaretii*	Y30/12	-	31.369156	
54	*Y. rohdei*	Y19/15	-	31.369781	
55	*Y. rohdei*	Y25/15	-	31.366907	
56	*Y. rohdei*	Y81/12	-	31.631624	
57	PTC	SLV-408 *Y. enterocolitica*	19.705093 19.517887	32.092827 31.95438	PCR 1 PCR 2
58	NTC	H_2O	-	31.190075 31.76178	PCR 1 PCR 2

Results, exclusivity

Cross reactivity	0
Total number	56
Exclusivity (%)	100 %

1.5.2 Results of PCR runs for determining the performance

Y. pestis (KIM5) (PHAS)

	Plate:	**1**		**2**		**3**		**4**		**5**		**Deviations**
	Date:	**151116**		**151117**		**151118**		**151119**		**151119**		
	Signature:	**MW**		**MW**		**MW**		**MW**		**MW**		
No	**GE/µL**	**Cq**	**Cq IAC**	**Cq**	**Cq IAC**	**Cq**	**Cq IAC**	**Cq**	**Cq IAC**	**Cq**	**Cq IAC**	
1	10^6	17.54	37.55									
2	10^6	17.51	38.65									
3	10^6	17.7	37.53									
4	10^6	17.39	38.22									
5	10^6	17.53	36.75									
6	10^6	17.56	36.41									
1	10^5	20.78	37.28									
2	10^5	20.76	34.76									

3	10^5	20.9	35.56									
4	10^5	20.77	35.08									
5	10^5	20.83	36.09									
6	10^5	20.91	35.26									
1	10^4	23.9	35.99									
2	10^4	23.91	35.3									
3	10^4	23.95	35.52									
4	10^4	23.89	35.61									
5	10^4	23.93	34.86									
6	10^4	23.99	35.47									
1	10^3	27.21	33.82									
2	10^3	27.15	34.17									
3	10^3	27.32	33.21									
4	10^3	27.24	34.93									
5	10^3	27.12	33.65									
6	10^3	27.42	34.19									
1	100	30.61	33.65									
2	100	30.57	32.35									
3	100	30.54	32.86									
4	100	30.25	33.69									
5	100	30.36	36.72									
6	100	30.78	33.19									
1	50	31.58	33.04	31.2	33.59	31.35	32.36	30.91	33.26	31.53	32.67	
2	50	32	32.7	30.91	36.82	30.61	35	30.85	32.7	31.46	32.93	
3	50	31.95	32.81	31.6	32.9	30.46	33.53	31.16	33.62	31.24	32.04	
4	50	31.5	33.2	30.91	33.49	30.42	32.94	30.8	32.77	31.38	33.53	
5	50	31.5	32.27	31.13	34.53	30.83	32.8	30.5	33.98	31.66	32.56	
6	50	31.72	33.23	31.2	32.59	30.93	32.76	30.73	32.85	32.2	32.87	
1	25	32.4	32.83	32.31	34.34	32	32.79	31.47	35.22	31.92	32.93	
2	25	32.37	33.12	31.74	34.21	31.94	33.69	31.85	33.61	32.49	32.79	
3	25	32.97	32.72	32.01	33.63	31.76	32.58	32.17	32.8	32.2	33.04	
4	25	32.53	32.98	31.94	33.53	31.74	33.91	31.24	33.56	32	33.48	
5	25	32.69	31.86	32.01	33.51	31.86	32.38	31.53	33.34	32.43	32.97	
6	25	32.34	33.34	32.53	33.27	32.01	32.53	32.54	33.5	32.81	32.87	
1	12,5	32.89	33.01	32.85	34.36	31.62	32.86	32.65	33.82	33.5	33.23	
2	12,5	32.68	32.24	32.98	33.8	32.51	34.4	32.17	33.68	33.57	31.96	
3	12,5	34.44	32.99	32.8	32.86	32.36	32.26	32.16	33.53	33.79	32.97	
4	12,5	33.75	32.91	33.46	33.25	31.71	32.32	32.28	33.45	33.19	33.16	
5	12,5	33.2	32.65	33.9	32.85	32.48	32.67	32.93	33.05	33.77	33.65	
6	12,5	33.35	33.23	32.84	33	34	34.54	32.4	34.22	33.68	32.85	
1	6,25	34.6	33.49	33.94	33.32	33.66	33.25	32.96	33.43	34.92	33.23	
2	6,25	34.75	32.82	34.84	34.25	33.8	32.57	33.23	34.4	33.63	32.67	
3	6,25	34.63	31.69	34.36	33.79	33.52	32.48	33.58	33.21	34.59	32.71	
4	6,25	34.45	32.85	33.32	33.66	33.54	32.37	33.86	33.67	33.15	32.45	
5	6,25	35.17	32.61	33.18	33.3	33.77	32.5	34.18	33.56	33.99	32.97	
6	6,25	33.68	32.63	34.95	33.48	33.02	32.8	33.81	33.79	34.14	33.69	

1	3,13	34.22	32.5	34.77	33.62	34.25	33.44	33.75	34.58	34.99	34.35	
2	3,13	34.64	32.86	35.35	33.58	-	32.83	33.8	34.25	36.39	32.52	
3	3,13	34.79	32.87	34.81	32.71	34.57	32.4	35	33.56	34.39	31.78	
4	3,13	37.66	33.65	34.55	34.38	-	32.56	34.48	33.42	35.19	31.84	
5	3,13	34.84	33.13	36.23	33.29	33.33	32.71	33.55	32.72	34.35	32.25	
6	3,13	36.63	32.85	34.79	33.53	36.71	32.62	34.56	33.48	35.48	32.39	
1	1,56	36.28	33.77	-	33.41	33.9	32.97	-	33.26	35.01	32.97	
2	1,56	-	31.84	-	33.61	33.98	33.58	35.46	33.21	-	32.44	
3	1,56	35.22	32.58	35.37	33.37	36.75	33.23	-	34.29	35.55	32.85	
4	1,56	-	33.04	36.36	33.37	36.66	32.69	34.31	33.39	34.98	32.77	
5	1,56	36.72	32.19	36.29	33.82	35.26	32.3	35.01	33.4	36.47	32.75	
6	1,56	36.7	32.2	36.27	34.19	-	32.22	35.73	34.19	-	32.56	
1	0,78	36.85	32.85	-	34.76	35.85	32.77	-	33.58	37.52	31.95	
2	0,78	36.67	32.24	36.31	34.29	34.86	32.57	34.98	33.34	35.55	32.49	
3	0,78	-	31.83	-	34.01	-	33.28	35.87	33.28	-	32.51	
4	0,78	35.73	32.32	-	33.54	-	32.48	-	33.7	-	32.38	
5	0,78	-	33.18	36.24	33.64	35.87	32.51	35.91	33.26	-	32.6	
6	0,78	35.74	32.81	-	34.69	-	32.38	34.76	33.52	-	32.78	
1	0,39	-	32.54	36.45	35.24	35.87	33.26	-	34.42	-	32.56	
2	0,39	36.72	33.19	-	33.76	-	32.35	-	33.34	-	33.23	
3	0,39	-	33.27	-	34.05	35.8	32.59	-	33.75	36.37	32.34	
4	0,39	-	32.17	-	33.55	-	33.23	35.85	33.37	-	33.86	
5	0,39	-	32.17	-	33.76	35.53	34.66	-	32.93	-	34.26	
6	0,39	-	31.92	35.8	34.72	-	33.72	35.88	32.81	-	31.98	
1	NTC	-	33.69	-	33.71	-	32.52	-	33.92	-	32.46	
2	NTC	-	32.03	-	33.85	-	32.78	-	33.45	-	32.65	
3	NTC	-	32.42	-	34.43	-	32.53	-	33.69	-	32.6	

Y. enterocolitica (SLV408) (SVA)

	Plate:	**1**		**2**		**3**		**4**		**5**		**Deviations**
	Date:	**151112**		**151117**		**151117**		**151117**		**151117**		
	Signature:	**JB**		**JB**		**JB**		**JB**		**JB**		
No	**GE/µL**	**Cq**	**Cq IAC**	**Cq**	**Cq IAC**	**Cq**	**Cq IAC**	**Cq**	**Cq IAC**	**Cq**	**Cq IAC**	
	10^6	20.1	32.7									
	10^6	19.9	31.8									
	10^6	19.9	31.6									
	10^6	19.9	31.3									
	10^6	19.9	30.9									
	10^6	19.9	30.5									
	10^5	22.9	30.0									
	10^5	22.9	29.6									
	10^5	22.8	29.8									
	10^5	22.8	29.7									
	10^5	22.8	29.7									
	10^5	22.9	29.8									
	10^4	26.1	29.5									
	10^4	26.0	29.5									
	10^4	26.2	29.4									

	10^4	26.0	29.5									
	10^4	26.0	29.4									
	10^4	26.1	29.7									
	10^3	29.4	29.9	29.6	30.3	29.8	30.5	29.3	30.6	28.9	29.9	
	10^3	29.3	29.8	29.7	30.4	29.8	30.7	29.2	30.6	28.9	30.1	
	10^3	29.4	29.8	29.6	30.2	29.8	30.5	29.3	30.5	28.9	30.0	
	10^3	29.3	29.8	29.7	30.3	29.8	30.5	29.3	30.5	28.7	29.9	
	10^3	29.3	29.9	29.6	30.3	29.7	30.5	29.3	30.7	28.8	29.9	
	10^3	29.2	29.9	29.7	30.1	29.8	30.6	29.2	30.4	28.8	30.0	
	100	32.2	30.0	32.3	30.7	32.6	30.9	32.3	30.7	32.0	30.4	
	100	32.5	30.0	32.2	30.3	32.7	30.8	32.1	30.8	31.6	30.2	
	100	32.7	29.9	32.7	30.5	32.4	30.5	32.1	30.6	31.8	30.4	
	100	32.6	30.0	32.8	30.5	32.8	30.7	31.7	30.9	31.8	30.3	
	100	32.3	29.9	32.4	30.6	32.4	30.7	32.1	30.8	31.9	29.9	
	100	32.4	29.9	32.9	30.4	32.7	30.8	32.1	30.7	31.8	30.0	
	50	33.8	30.6	33.4	30.6	33.0	30.7	33.2	30.8	32.4	30.4	
	50	33.9	30.6	33.2	30.8	33.3	30.9	33.3	30.7	32.9	30.4	
	50	33.6	30.5	33.6	30.6	33.9	30.9	33.4	30.8	32.9	30.3	
	50	34.1	30.5	33.4	30.7	33.9	30.7	32.9	30.6	32.5	30.4	
	50	33.9	30.4	33.4	30.8	33.3	30.8	33.0	30.7	32.8	30.1	
	50	33.6	29.9	33.5	30.6	33.5	30.9	33.6	30.7	33.1	30.2	
	25	34.2	29.9	34.4	30.9	34.7	30.8	34.1	30.7	33.5	30.6	
	25	34.8	30.0	34.7	30.9	34.9	30.8	34.1	30.9	33.9	30.5	
	25	34.8	29.9	34.9	30.7	34.3	30.8	34.1	30.9	33.3	30.3	
	25	33.7	29.9	35.7	30.7	34.8	30.7	34.9	30.7	33.5	30.5	
	25	34.3	29.9	34.6	30.8	34.0	30.8	33.5	30.7	33.4	30.5	
	25	34.3	29.9	35.3	30.7	33.9	30.9	33.9	30.7	33.5	30.4	
	12.5	35.3	30.0	36.0	30.6	35.4	30.7	34.9	31.1	36.9	30.2	
	12.5	34.9	29.9	35.7	30.8	35.9	30.9	34.3	30.7	35.5	30.3	
	12.5	34.5	29.9	35.8	30.6	36.2	30.8	34.3	30.8	34.4	30.4	
	12.5	35.5	30.0	35.4	30.8	35.7	30.9	36.1	30.9	34.4	30.4	
	12.5	35.6	30.0	34.8	30.7	36.1	30.8	34.8	30.9	34.9	30.4	
	12.5	35.9	30.0	35.6	30.7	35.2	30.9	34.9	30.8	35.1	30.4	
	6.25	35.5	30.0	37.9	30.6	36.5	30.7	36.9	30.7	36.9	30.4	
	6.25	35.4	29.9	36.2	30.6	36.5	30.7	36.4	30.7	35.9	30.4	
	6.25	35.9	29.9	36.8	30.6	35.1	30.7	35.2	30.7	35.0	30.4	
	6.25	36.0	30.0	37.8	30.7	36.4	30.7	35.2	30.6	35.8	30.4	
	6.25	36.4	29.9	-	30.6	37.2	30.8	35.9	30.7	35.0	30.4	Trouble with tips
	6.25	37.9	30.2	-	30.6	37.9	30.8	35.8	30.7	34.6	30.3	Trouble with tips
	3.125	37.8	30.2	-	30.4	-	30.9	35.9	30.8	-	30.3	
	3.125	-	30.0	36.9	30.7	37.1	30.7	37.4	30.7	-	30.3	
	3.125	36.7	30.0	36.7	30.7	38.1	30.8	37.3	30.7	36.0	30.4	
	3.125	37.8	30.1	37.8	30.8	37.9	30.8	36.3	30.8	35.9	30.6	
	3.125	36.3	30.1	37.8	30.4	36.3	30.7	-	30.8	35.2	30.4	
	3.125	37.1	30.1	37.9	30.7	36.9	30.9	36.4	30.6	36.0	30.4	
	1.56	-	29.9	-	30.7	38.1	30.7	35.9	30.9	-	30.3	
	1.56	37.8	30.2	37.9	30.9	36.8	30.8	36.7	30.8	-	30.7	
	1.56	-	30.0	-	30.9	38.1	30.7	-	31.0	-	30.9	
	1.56	-	29.9	36.5	30.8	38.1	30.7	35.6	30.6	-	30.8	
	1.56	-	29.9	38.1	30.8	38.1	30.8	37.4	30.7	-	30.9	
	1.56	37.8	29.9	-	30.6	-	30.8	37.5	30.6	36.5	30.8	
	0.78	-	29.9	-	30.6	-	30.9	-	30.8	36.8	30.2	
	0.78	37.9	30.0	-	30.7	37.9	30.8	-	30.9	-	30.4	
	0.78	-	30.0	-	30.5	37.9	30.7	-	30.7	37	30.4	

	0.78	-	29.9	-	30.6	37.2	30.9	-	30.6	-	30.3	
	0.78	-	29.8	-	30.6	-	30.7	-	30.8	-	30.5	
	0.78	-	29.9	-	30.6	-	30.7	-	30.6	37.1	30.3	
	0.39	38.1	29.9	-	30.4	-	30.8	-	30.7	-	30.2	
	0.39	-	30.0	-	30.5	-	30.6	-	30.5	-	30.4	
	0.39	-	30.0	-	30.5	-	30.7	-	30.6	37.0	30.3	
	0.39	-	30.0	-	30.8	-	30.7	-	30.8	-	30.5	
	0.39	-	30.0	37.9	30.5	-	30.7	-	30.5	-	30.1	
	0.39	-	29.9	-	30.9	-	30.6	37.6	30.7	-	30.7	
	NC	-	29.5	-	29.9	-	30.3	-	30.2	-	30	
	NC	-	29.6	-	30.1	-	30.1	-	30.2	-	30	
	NC	-	29.5	-	30.1	-	30.1	-	30.1	-	30.2	

LOD

Run	Result LOD (GE/reaction) PHAS	Result LOD (GE/reaction) SVA
1	3.13	6.25
2	3.13	6.25 (trouble with tips)
3	6.25	6.25
4	3.13	6.25
5	3.13	6.25
Final LOD	**6.25**	**6.25**

Efficiency

Run: 151116 (run 1)

Parameters	Result PHAS	Result SVA
Gradient	-3.2707	-3.2092
R^2	0.9997	0.999
Confidence interval for E	+/- 0.01190	+/- 0.00072
Efficiency (E)	1.021831449	1.049293385

Precision: Repeatability

Concentration (GE/reaction)	Average (Cq)	Standard deviation (STDAV)	Coefficient of variation (CV)
PHAS			
3.13	35.46	1.24	3.50
6.25	34.55	0.45	1.30
12.5	33.39	0.58	1.74
SVA			
3.13	37.26	0.66	1.77

6.25	36.60	0.85	2.32
12.5	35.75	0.36	1.01

Precision: Reproducibility

Run	Parameters
This has unfortunately not been able to be carried out within the framework of the project due lack of time.	

Robustness

Risk analysis
This validation evaluates only the performance of the method in analysis of pure DNA. When analysing samples with suspected presence of *Y. pestis*, *pseudotuberculosis* and *enterocolitica*, such as in clinical or environmental samples, the properties of the matrix must be taking into account. The matrix may for example inhibit the DNA polymerase. The evaluation should therefore be repeated in the presence of the relevant matrix.

Summary

Performance	Result PHAS/SVA	Meets the requirements?
Inclusivity	100 %	Yes
Exclusivity	100 %	Yes
LOD	6.25 / 6.25	Yes
Efficiency	102 % / 105 %	Yes
Precision: Repeatability	1.30 / 2.32	Yes
Precision: Reproducability	-	-
Robustness	Estimated	Ok

Conclusion

The specificity of this PCR is adequate (100%). Because that the definition of non-pathogenic *enterocolitica* strains is very diffuse the PCR was designed to detect all strains of this species. For *pseudotuberculosis* there are two unusual serotypes (O: 11 and O: 12). In previous studies (2) these serotypes have been difficult to detect due to that they differ from other serotypes. It is unclear whether this PCR can identify these serotypes or not.

1.6 REFERENCES

1 Reuter *et al*., (2014) Parallel independent evolution of pathogenicity within the genus *Yersinia*. *PNAS* 111(18):6768-6773

2 Lambertz, Nilsson and Hallanvuo, (2008) *TaqMan-based real-time PCR method for detection of Yersinia pseudotuberculosis in food*. Applied and Environmental Microbiology 74(20):6465-6469

3 Sabina *et al*., (2011) *Yersinia enterocolitica*: Mode of Transmission, Molecular Insights of Virulence, and Pathogenesis of Infection. Journal of pathogens 2011 (Article ID 429069)

FORUM FÖR
BEREDSKAPSDIAGNOSTIK

FBD

FBD 005-2
2016-10-12

1 PROTOCOL FOR VALIDATION OF A REAL-TIME PCR ASSAY FOR DETECTION OF BACTERIA

Name of the method
Probe-based real-time PCR for detection of *Brucella melitensis.*

Summary of results		
Specificity, inclusivity (%)	100 %	
Specificity, exclusivity (%)	100 %	
Efficiency (%) and CI	112%	0,993
LOD (GE/reaction)	6,25	
Precision, repeatability (CV %)	0,61	
Precision, reproducibility (CV %)	1,49	-
Robustness:	Evaluated	Evaluated

This PCR method for the detection of *Brucella melitensis* has showed sufficient specificity as it can detect all tested strains of *B. melitensis* and no other agents. Above all, it runs against other species of *Brucella* negative, which is the major challenge since *Brucella* family consists of equally genetically similar species.
The method has good precision both in terms of repeatability (results within a single run) and reproducibility (results from 5 different runs and performed by different laboratory technicians). The method has also been used in a blinded ring-trial and gave accurate results. This validation was performed with extracted DNA from colonies. To investigate how well the method performs in presence of inhibitors further evaluation with different matrices is needed.

2016-10-10	**Tara Wahab and Sevinc Ferrari**	
Date	**Performed by**	**Approved by**

1.1 BACKGROUND AND AIM

Aim

To develop a new real-time PCR to detect all biovars of *Brucella melitensis*.

Background

Previously, there has not been a real-time PCR method that simultaneously can detect all biovars of *Brucella melitensis*.

Parameters to be validated

☒ Specificity
☒ LOD
☒ Efficiency
☒ Precision: repeatability
☒ Precision: reproducibility
☒ Accuracy
☒ Robustness

Associated protocols

FBD 004 – Protocol for preparation of primers and probes

Used ☐ Yes ☒ No

The software, SmiPrimer (developed by Erik Alm and others) was used to develop and optimize primers and probes.

FBD 007 – Protocol for preparation of reference and control material (DNA)

Used ☐ Yes ☒ No

We used extracted DNA from seal herpes cultures as an internal positive control,

Protocols used for validation:

Name	FBD or other	Agency (if other)
FBD 005-2	FBD	

1.2 MATERIALS AND METHODS

B.melitensis

Primers and probes

Name of oligos	Sequence	Manufacturer	Batch number
Bmel-F	5'- GCTCGACACAAAGGGCCA-3'	Biomers	00214190-3
Bmel-R	5'-CAAGCGTGGTCTGGCGA-3'	Biomers	00214190-4
Bmel-P	FAM-CCGAGATACAAA-MGB	Biomers	4316034

Name of internal controls	Sequence	Manufacturer	Batch number
IAC_F	5'- GGGCGAATCACAGATTGAATC-3'	Eurofins Genomics	18457674-F6
IAC_R	5'- GCGGTTCCAAACGTACCAA-3'	Eurofins Genomics	18457673-B2
IPCP-VIC	VIC-TTTTTATGTGTCCGCCACCATCTGGATC-MGB	Applied Biosystems/Lifetech	101608-D8

Control DNA

Strain	Batch	Extraction method	Measurement	Concentration
B. melitensis ATCC 23456	-	EZ1	Qubit	7,44 ng/µl

Positive DNA control and internal control

Control	Description	Batch number or date for manufacturing	Thawed (date)
Positive	B. melitensis ATCC 23456		
IAC	DNA from Phocine Herpesvirus 1 (PhHv-1)		
NC	DNAse and RNAase free Water		

Mastermix

Reagents	Name	Manufacturer	Batch number	Concentra-tion (stock)	Volume (µl)
H2O	-	Sigma	RNBC8414	-	5,05
Mastermix*	2x PerfeCta Multiplex qPCR SuperMix	Applied Biosystems®	23076		12,5
Primer 1:	Bmel-F	See above	See above	0.9 µM	1,0 (primerprobmix)
Primer 2:	Bmel-R	See above	See above	0.9 µM	
Prob 1	Bmel-P	See above	See above	0.2 µM	

Control primer 1(IAC):	IACF	See above	See above	10 µM	1,25 (primer-probe mix)
Control primer 2(IAC):	IACR	See above	See above	10 µM	
Internal control template (IAC):	IPCP-VIC	See above	See above	2 µM	
Template					5
Total					**25**

Oligos (stock solution)

Stock solution	Manufacturing date	Thawed (times)
OM mel short	2016-05-09	Same tube has been consistently used, not frozen and thawed.

PCR-program

Program	Temp (°C)	Time (min)	
Initial denaturation	95 °C	3 min	
Cycling			**Number of cycles**
Denaturation of DNA	95 °C	0:03	
Hybridisation and elongation	60 °C	0:30	45

Instruments

Instrument	Model	Manufacture	Service of instrument
Real-time PCR-mashine	StepOne Plus real-time PCR system, ABI 7500 (FAST)	Applied Biosystems®	Real-time PCR-mashine
Extraction robot	**EZ1**	**Qiagen**	Extraction robot

Number of strains exclusivity panel: 76 (of which 25 are other species of *Brucella* spp)
Number of strains inclusivity panel: 6 *B. melitensis* reference strains.
In addition to the strains of the panel 120 patient isolates, which were typed using other methods, were analyzed by this PCR method.
The preferred concentration of materials: 2 ng /µl (10ng/reaction).
If other concentration used, specify which: Certain strains of exclusivity panel had lower concentration than 2 ng/µl. The lowest concentration was 0.17 ng/µl.

1.3 PREDEFINED SPECIFICATIONS

Recommended performance requirements

Specificity requirements (inclusivity and exclusivity):
100 %

Minimum acceptable value for Efficiency, E (%):
Between 90 and 110 %

Limit of detection, LOD (GE/µl):
Currently there are no defined demands on the LOD.

1.4 BENCHMARKS

Controls

Control	Cq (average)	Standard deviation
Negative PCR control (NC)	-	-
Internal amplification control (IAC)	~32	-
Positive PCR control	-	-

Recommended values for dilution series

Dilution serie was done	
LOD (GE/reaction and Cq)	-
Dilutions (GE/reaction)	From 10^6 to 0,039 (GE/reaction)

1.5 RESULTS AND CALCULATIONS

1.5.1 Specificity

Inclusivity panel

No	Agents	Strain info	*B. mel*– PCR Cq	Deviations
1	*B. melitensis*	ATCC 23456	16,68	
2	*B. melitensis*	NCTC 10094	16,79	
3	*B. melitensis*	NCTC10508	16,92	
4	*B. melitensis*	NCTC 10509	16,93	
5	*B. melitensis*	16M	14,14	
6	*B. melitensis*	2065	17,2	

Result, inclusivity panel

Identified	6
Total number	6
Inclusivity (%)	100

Additionally, all (120) patient isolates could be detected by this Real-Time PCR. They were not included in the inclusivity panel because typing was not made by sequencing but by MALDI-TOF or MLVA. The isolates would be needed to be further characterized by sequencing before they can be included in a panel, but this result is still interesting for Real-Time PCR specificity of the method.

Exclusivity panel

No	**Agents**	**Strain information**	**Target 1 Cq:**	**Target 2 Cq:**	**IAC Cq**	**Deviations**
1	*Actinomyces pyogenes*	CCUG 13230	-	-	32,70	
2	*Alcaligenes denitrificans*	CCUG 407	-	-	32,57	
3	*Bacillus cereus*	CCUG 7414	-	-	32,48	
4	*Bacillus subtilis*	ATCC 6633	-	-	32,67	
5	*Bordetella bronchiseptica*	CCUG 219	-	-	32,53	
6	*Enterococcus fecalis*	ATCC 29212	-	-	32,43	
7	*Erysipelotrix rhusiopatiae*	CCUG 221	-	-	32,52	
8	*Escherichia coli*	ATCC 35218	-	-	32,49	
9	*Klebsiella oxytoca*	CCUG 15717	-	-	32,75	
10	*Klebsiella pneumoniae*	CCUG 225	-	-	32,56	
11	*Listeria monocytogenes*	CCUG 15527	-	-	32,51	
12	*Nocardia asteroides*	CCUG 10073	-	-	32,74	
13	*Pasteurella multocida*	CCUG 229	-	-	32,43	
14	*Pasteurella pneumotropica*	CCUG 12398	-	-	32,63	
15	*Proteus mirabilis*	CCUG 26767	-	-	32,50	
16	*Pseudomonas aeruginosa*	CCUG 17619	-	-	32,51	
17	*Rhodococcus equi*	CCUG 892	-	-	32,88	
18	*Salmonella Dublin*	CCUG 35631	-	-	32,56	
19	*Salmonella Thyphimurium*	CCUG 31969	-	-	32,90	
20	*Salmonella Zanzibar*	CCUG 41921	-	-	32,55	
21	*Staphylococcus aureus*	CCUG 4151	-	-	32,80	
22	*Staphylococcus intermedius*	CCUG 49053	-	-	32,70	
23	*Streptococcus agalactiae*	CCUG 39325	-	-	32,54	
24	*Streptococcus dysgalactiae*	CCUG 27436	-	-	32,80	
25	*Streptococcus equi*	CCUG 27367	-	-	32,57	
26	*Streptococcus pyogenes*	CCUG 12701	-	-	32,55	
27	*Streptococcus uberis*	CCUG 27444	-	-	32,56	
28	*Streptococcus zooepidemicus*	CCUG 23256	-	-	32,61	
29	*Yersinia enterocolitica*	CCUG 8239	-	-	32,26	
30	*Yersinia pseudotuberculosis*	CCUG 5855	-	-	32,28	
31	*Fusobacterium necrophorum*	CCUG 9994	-	-	32,73	
32	*Clostridium perfringens*	CCUG 1795	-	-	32,65	
33	*Bacteroides fragilis*	ATCC 25285	-	-	32,44	
34	*Taylorella equigenitalis*	CCUG 16464	-	-	32,41	
35	*Actinobacillus pleuropneumoniae*	CCUG 12837	-	-	32,44	
36	*Haemophilus influenzae*	ATCC 49247	-	-	32,61	
37	*Haemophilus somnus*	CCUG 28029	-	-	32,52	

38	*Streptobacillus moniliformis*	CCUG 33440	-	-	32,39	
39	*Bacillus anthracis*	NCTC1328	40,18	-	32,45	No typical curve. The analysis was run on six replicates and one of them came up at the Cq 40. These results are considered negative.
40	*Burkholderia mallei*	NCTC120	-	-	32,66	
41	*Burkholderia pseudomallei*	NCTC8707	-	-	32,48	
42	*EHEC*	EDL333	-	-	32,38	
43	*Yersinia pestis*	570-04	-	-	32,23	
44	*Ochrabactrum anthropi*	ATCC 49188	-	-	32,39	
45	*Francisella tularensis typ A*	FSC237	-	-	32,53	
46	*Francisella tularensis typ B*		-	-	32,59	
47	*B. abortus*	ATCC 23448	-	-	36,02	
48	*B. abortus*	544	-	-	38,83	
49	*B. abortus*	NCTC 00624	-	-	36,31	
50	*B. abortus*	NCTC10501	-	-	35,98	
51	*B. abortus*	NCTC10502	-	-	37,94	
52	*B. abortus*	NCTC10503	-	-	34,79	
53	*B. abortus*	NCTC10504	-	-	35,05	
54	*B. abortus*	NCTC10505	-	-	37,3	
55	*B. abortus*	NCTC10506	-	-	35,98	
56	*B. abortus*	NCTC10507	-	-	36,72	
57	*B. canis*	ATCC 23365	-	-	38,13	
58	*B. canis*	NCTC 10854	-	-	35,98	
59	*B. canis*	3.4.2008/122	-	-	36,46	
60	*B. canis*	E20140122-106	-	-	38,62	
61	*B. ceti*	NCTC 12891	-	-	36,7	
62	*B. inopinata*	CAPM 6436	-	-	35,69	

63	*B. microti*	CAPM 6434	-	-	37,95	
64	*B. neotomae*	ATCC 23459	-	-	35,28	
65	*B. ovis*	ATCC 25840	-	-	34,91	
66	*B. ovis*	NCTC 10512	-	-	35,01	
67	*B. pinnipedialis*	NCTC 12890	-	-	36,3	
68	*B. suis*	ATCC 23444	-	-	38,42	
69	*B. suis*	NCTC 10316	-	-	35,26	
70	*B. suis*	NCTC 12042-01	-	-	36,97	
71	*B. suis*	NCTC 10510	-	-	35,68	
72	*B. suis*	NCTC 10511	-	-	36,04	
73	*B. suis*	NC 10385-02	-	-	35,82	
74	*B. suis*	NCTC 11996	-	-	36,83	
75	*B. suis*	1720	-	-	38,96	
76	*B. suis*	1030	-	-	38,38	

Results, exclusivity panel

Crossreactivity	0
Total number	76
Exclusivity (%)	100%

1.5.2 Results of PCR runs

Effeciency

Run:

Parameters	Results
Slope	-3,055
R^2	0,993
Effectivity (E)	**112 %**

Precision:

Concentration (GE/reaction)	Average (Cq)	Standard deviation (SD)	Variation cofficient (CV)
5	35,402	0,377	1,064
2,5	36,395	0,46	1,26
1,25	37,415	0,231	0,61

Precision: Reproducibility

Run	Parameters
This has unfortunately not been able to be carried out within the framework of the project due lack of time.	

Robustness

Risk analysis
This validation evaluates only the performance of the method in analysis of pure DNA. When analyzing samples with suspected presence of *B. melitensis*, such as in clinical or environmental samples, the properties of the matrix must be taking into account. The matrix may for example inhibit the DNA polymerase. The evaluation should therefore be repeated in the presence of the relevant matrix.

Limit of detection

As a final LOD determines the maximum LOD score obtained from all runs. Final LOD for this PCR is 1.25 GE/uL (= 6.25 GE/reaction) where all six replicates in all runs are positive. The yellow marked run was run by another analyst.

DNA conc.	Run 1	Run 2	Run 3	Run 4	Run 5
40 GE/µl	6	6	6	6	6
20 GE/µl	6	6	6	6	6
10 GE/µl	6	6	6	6	6
5 GE/µl	6	6	6	6	6
2,5 GE/µl	6	6	6	6	6
1,25 GE/µl	6	6	6	6	6
0,625 GE/µl	6	6	6	6	5 of 6
0,312 GE/µl	6	6	**3 of 6***	6	5 of 6
0,156 GE/µl	5 of 6	6	5 of 6	6	4 of 6
0,078 GE/µl	4 of 6	4 of 6	1 of 6	5 of 6	4 of 6

Summary

Performance	Result	Meets the requirements?
Inclusivity	100 %	Yes
Exclusivity	100 %	Yes
LOD	6,25	Yes
Efficiency	112 %	Yes
Precision: Repeatability	0,61	Yes
Precision: Reproducability	1,49	Yes
Robustness	Estimated	Ok

Conclusion

The specificity of this PCR is 100%. The exclusivity and inclusivity test for this *B. melitensis* real-time PCR method is well for the strains included in the study. The method is now used daily at the Public Health Agency for the primary diagnosis of *B. melitensis*.

1.6 REFERENCES

Projektrapport: harmonisering av odling och PCR detektion av Brucella. FBD 2014/14.

Kvalitetssäkring av realtids-PCR samt laboratoriesäkerhet för analys av högpatogena bakterier inom Forum för Beredskapsdiagnostik. FBD 2014/15.

FORUM FÖR
BEREDSKAPSDIAGNOSTIK

FBD

1 PROTOCOL FOR VALIDATION OF A REAL-TIME PCR ASSAY FOR DETEKTION OF BACTERIA

Name of the method
Real-time PCR for detection of *Brucella suis*.

Summary of results
Specificity, inclusivity: 89% (Detected 4 of 5 biovars, 8 out of 9 strains of tested *B. suis*)
Specificity, exclusivity: 100% (The PCR method didn't detect any of the 73 other DNA than *Brucella suis* tested)
Efficiency (%) och CI: 113% (0,99)
LOD (GE/reaction) 6,25
Robustness: The PCR method was run at two different laboratories with two different PCR mashines. The same results were obtained which means that the PCR method is robust.

Summary of the results from the validation:
The PCR method is robust and works well for 4 (bv 1- bv 4) out of 5 *B. suis* biovars. This PCR method has the best primer-probe combination out of five that were tested during the project. Even though all species of the genus *Brucella* are genetically very similar no false positive results were obtained. This validation was done with pure DNA from colony. In order to investigate how well the method performs in presence of possible inhibitors or other interferences additional studies should be carried out with different matrices.

2016-10-12	***Tara Wahab, Sevinc Ferrari***	
Date	**Performed by**	**Approved by**

1.1 BACKGROUND AND AIM

Aim:
To be able to detect all biovars of *Brucella suis* with one real-time PCR assay.

Background:
B. suis is an important pathogen usually found in feral pigs and hares. It can cause severe disease in humans and therefore it is of great importance that there is a real-time PCR method that can detect all *B. suis* biovars simultaneously. See FBD 21, 2016.

Parameters to be validated

X Specificity
X LOD
X Efficiency
X Robustness

Associated protocols

FBD 004 - Protocol for preparation of primers and probes
Used ☐ Yes x No
Primers and probes were designed by Erik Alm and Tara Wahab at the Swedish Public Health Agency (FOHM) by using Smidesigner developed by Erik Alm. Four primer-probe combinations were designed and none of them were 100 % specific, showing cross reactivity with other *Brucella* sp. A new real-time PCR, recently published by Hänsel et al (2015) , that can detect 4 out of 5 biovars of *B. suis* was set up at the our institutes and used in the validation.

FBD 007 – Protocol for preparation of reference and control material (DNA)
Used ☐ Yes x No
In order to measure the efficiency and LOD of this real-time PCR method *Brucella suis* ATCC 23444 was used as reference DNA.

Protocols used for validation:
The dilutions were done according to figure 1 in the project report ”Kvalitetssäkring av Realtids-PCR samt laboratoriesäkerhet för analys av högpatogena bakterier inom Forum för Beredskapsdiagnostik.FBD” By Moa Lavander, Talar Boskani, Edvin Karlsson, Marie Karlsson, Paula Ågren and Sara Åkerström. 2014/15.

1.2 MATERIALS AND METHODS

Primers and probes

Name of oligos	Sequence	Manufacture	Batch number
Bsuis5-F	GCCAAATATCCATGCGGGAAG	Biomers	00220299-1
Bsuis5-R	TGGGCATTCTCTACGGTGTG	Biomers	00220299-2
Bsuis5-P	FAM-TTGCGCTTTTGTGATCTTTGCGCTTTATGG-TAMRA	Biomers	00220299-3

Name of internal controls	Sequence	Manufacture	Batch number
IAC_F	5'- GGGCGAATCACAGATTGAATC-3'	Eurofins Genomics	18457674-F6
IAC_R	5'- GCGGTTCCAAACGTACCAA-3'	Eurofins Genomics	18457673-B2
IPCP-VIC	VIC-TTTTTATGTGTCCGCCACCATCTGGATC-MGB	Applied Biosystems/Lifetech	101608-D8

Control DNA

Strain	Batch/reference	Extraction method	Measurement	Concentration
B. suis ATCC 23444	-	EZ1	Qubit	11 ng/µl

Positive DNA control and internal control

Control	Description	Batch nr. or date for manufacturing
Positive control	B. suis ATCC 23444	-
IAC	DNA from seal herpesvirus typ 1 (PhHv-1)	-
NC	DNAse and RNAase free Water	-

Master mix

Reagents	Name	Manufacturer	Batch no.	Concentration	Volume (µl) /reaction
H_2O	-	Sigma	RNBC8414	-	5,05
Mastermix*	2x PerfeCta Multiplex qPCR SuperMix	Applied Biosystems®	23076		12,5
Primer 1:	Bsuis5-F	See above	See above	0.9 µM	1,0 (primer-probe mix)
Primer 2:	Bsuis5-R	See above	See above	0.9 µM	
Probe 1	Bsuis5-P	See above	See above	0.2 µM	

Control primer 1(IAC):	IACF	See above	See above	10 µM	1,25 (primer-probe mix)
Control primer 2(IAC):	IACR	See above	See above	10 µM	
Probe control (IAC):	IPCP-VIC	See above	See above	2 µM	
Internal control template (IAC):	Phocine Herpesvirus 1 (PhHv-1)	-	-	Cq ~32	0,2
Template	B. suis ATCC 23444				5,0
Total					25,0

Oligos (stock solution)

Stock solution	Manufacturing date	Thawed (times)
OM suis 5		The same solution was used without freeze-thawing

PCR-program

Program	Temp (°C)	Time (min)	
Initial denaturation	95 °C	3 min	
Cycling			**Number och cycles**
Denaturation of DNA	95 °C	3 sec	
Hybridisation and elongation	60 °C	30 sec	45

Instruments

Instrument	Model	Manufacturer	Service of instrument
PCR machine	StepOne Plus real-time PCR system, ABI 7500 (FAST)	Applied Biosystems®	2016-04-01
Extraction robot	**EZ1**	**Qiagen**	

Specificity – exclusivity

Number of strains in the exclusivity panel: 73 (Among these 27 strains are of other species than *Brucella suis*)

Number of strains in the inclusivity panel: 9 *B. suis* reference strains.

Recommended koncentration: 2 ng/µl (10ng/reaction).

If other concentrations are used, please state: Some strains in the panel had lower concentrations than 2 ng/µl. The lowest koncentration was 0,17 ng/µl.

1.3 PREDEFINED SPECIFICATIONS

Recommended performance requirements

Specificity requirements (inclusivity and exclusivity): 100%

Minimum acceptable value for Efficiency, E (%): 90%

Limit of detection, LOD (GE/µl): 1,25 GE/reaction

1.4 BENCHMARKS

Controls

Control	Cq (average)	Standard deviation
Negative PCR-control (NC)	-	-
Internal amplification control (IAC)	~32	-
Positive PCR control	-	-

Recommended values for dilution series

Dilution series	
LOD (GE/reaction and Cq)	We have done 10 fold dilutions from 10^6 Cq 19 to 100 Cq 31 GE/reaction and then two fold dilutions to 0,39 GE/reaction.
Dilutions (GE/reaction)	10^6 – 0,39

1.5 RESULTS AND CALCULATIONS

1.5.1 Specificity

Exclusivity

No	Agents	Strain info	PCR 1 Cq:	PCR 2 Cq:	IAC Cq	Deviations
1	Actinomyces pyogenes	CCUG 13230	-	-	31,49	
2	Alcaligenes denitrificans	CCUG 407	-	-	31,46	
3	Bacillus cereus	CCUG 7414	-	-	31,46	
4	Bacillus subtilis	ATCC 6633	-	-	31,43	
5	Bordetella bronchiseptica	CCUG 219	-	-	31,58	
6	Enterococcus fecalis	ATCC 29212	-	-	31,64	
7	Erysipelotrix rhusiopatiae	CCUG 221	-	-	31,53	
8	Escherichia coli	ATCC 35218	-	-	31,59	
9	Klebsiella oxytoca	CCUG 15717	-	-	31,63	
10	Klebsiella pneumoniae	CCUG 225	-	-	31,32	
11	Listeria monocytogenes	CCUG 15527	-	-	31,46	
12	Nocardia asteroides	CCUG 10073	-	-	31,34	

13	Pasteurella multocida	CCUG 229	-	-	31,50	
14	Pasteurella pneumotropica	CCUG 12398	-	-	31,36	
15	Proteus mirabilis	CCUG 26767	-	-	31,49	
16	Pseudomonas aeruginosa	CCUG 17619	-	-	31,47	
17	Rhodococcus equi	CCUG 892	-	-	31,55	
18	Salmonella Dublin	CCUG 35631	-	-	31,46	
19	Salmonella Thyphimurium	CCUG 31969	-	-	31,42	
20	Salmonella Zanzibar	CCUG 41921	-	-	31,55	
21	Staphylococcus aureus	CCUG 4151	-	-	31,43	
22	Staphylococcus intermedius	CCUG 49053	-	-	31,43	
23	Streptococcus agalactiae	CCUG 39325	-	-	31,42	
24	Streptococcus dysgalactiae	CCUG 27436	-	-	31,40	
25	Streptococcus equi	CCUG 27367	-	-	31,52	
26	Streptococcus pyogenes	CCUG 12701	-	-	31,52	
27	Streptococcus uberis	CCUG 27444	-	-	31,69	
28	Streptococcus zooepidemicus	CCUG 23256	-	-	31,60	
29	Yersinia enterocolitica	CCUG 8239	-	-	31,46	
30	Yersinia pseudotuberculosis	CCUG 5855	-	-	31,48	
31	Fusobacterium necrophorum	CCUG 9994	-	-	31,66	
32	Clostridium perfringens	CCUG 1795	-	-	31,55	
33	Bacteroides fragilis	ATCC 25285	-	-	31,55	
34	Taylorella equigenitalis	CCUG 16464	-	-	31,49	
35	Actinobacillus pleuropneumoniae	CCUG 12837	-	-	31,59	
36	Haemophilus influenzae	ATCC 49247	-	-	31,48	
37	Haemophilus somnus	CCUG 28029	-	-	31,49	
38	Streptobacillus moniliformis	CCUG 33440	-	-	31,54	
39	Bacillus antracis	NCTC1328	41,56	-	31,44	Not a typical curve. It’s neg.
40	Burkholderia mallei	NCTC120	-	-	31,43	
41	Burkholderia pseudomallei	NCTC8707	-	-	31,48	
42	EHEC	EDL333	-	-	31,44	
43	Yersinia pestis	570-04	-	-	31,35	
44	Ochrabactrum anthropi	ATCC 49188	-	-	31,33	
45	Francisella tularensis typ A	FSC237	-	-	31,55	
46	Francisella tularensis typ B		-	-	31,44	
47	*B. abortus*	ATCC 23448	-	-	40,63	
48	*B. abortus*	544	-	-	42,25	
49	*B. abortus*	NCTC 00624	-	-	40,83	
50	*B. abortus*	NCTC10501	-	-	36,55	

51	*B. abortus*	NCTC10502	-	-	38,11	
52	*B. abortus*	NCTC10503	-	-	38,24	
53	*B. abortus*	NCTC10504	-	-	37,36	
54	*B. abortus*	NCTC10505	-	-	37,31	
55	*B. abortus*	NCTC10506	-	-	39,43	
56	*B. abortus*	NCTC10507	-	-	38,97	
57	*B. canis*	ATCC 23365	-	-	42,02	
58	*B. canis*	NCTC 10854	-	-	43,58	
59	*B. canis*	3.4.2008/122	-	-	36,04	
60	*B. canis*	E20140122-106	-	-	35,94	
61	*B. ceti*	NCTC 12891	-	-	39,03	
62	*B. inopinata*	CAPM 6436	-	-	37,49	
63	*B. microti*	CAPM 6434	-	-	43,76	
64	*B. neotomae*	ATCC 23459	-	-	36,89	
65	*B. ovis*	ATCC 25840	-	-	40,77	
66	*B. ovis*	NCTC 10512	-	-	41,64	
67	*B. pinnipedialis*	NCTC 12890	-	-	38,37	
68	*B. melitensis*	ATCC 23456	-	-	43,81	
69	*B. melitensis*	NCTC 10094	-	-	38,32	
70	*B. melitensis*	NCTC10508	-	-	40,06	
71	*B. melitensis*	NCTC 10509	-	-	40,08	
72	*B. melitensis*	16M	-	-	40,5	
73	*B. melitensis*	2065	-	-	43,81	

Results, exclusivity

Cross reactivity	0

Total number	73
Exclusivity (%)	100

Inclusivity

No	Agents	Strain info	*B. suis*–PCR Cq	Deviations
1	*B suis*	ATCC 23444	15.94 & 15.95	
2	*B suis*	NCTC 10316	17.36 & 17.46	
3	*B suis*	NCTC10510	36.95 & 39.45	
4	*B suis*	NCTC 10511	16.33 & 16.47	
5	*B suis*	NC10385-02	16.68 & 16.78	
6	*B suis*	NC12042-01	16.46 & 16.58	
7	*B suis*	NCTC11996	-	
8	*B suis*	1720	13,78 & 14,01	
9	*B suis*	1030	13,23 & 13,34	

Results, inclusivity

Identified	8
Total number	9
Inclusivity (%)	89%

1.5.2 Results of PCR runs for determining the performance

The table below shows the PCR runs for *B. suis* ATCC 23444 validation with the following concentrations: 10^6, 10^5, 10^4, 10^3, 10^2, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39 genome equivalent (GE) per reaction. Run nr 5 was carried out by a different laboratory worker.

LOD

The final LOD for the new PCR is 1,25 GE/µl where all six replicates in all runs were positive. Run 5 was carried out by another laboratory worker.

Dilution series DNA konc.	Run 1	Run 2	Run 3	Run 4	Run 5
40 GE/µl	6	6	6	6	6
20 GE/µl	6	6	6	6	6
10 GE/µl	6	6	6	6	6
5 GE/µl	6	6	6	6	6
2,5 GE/µl	6	6	6	6	6

1,25 GE/µl	6	6	6	**was missed***	6
0,625 GE/µl	6	4/6	6	6	6
0,312 GE/µl	5/6	5/6	4/6	6	5/6
0,156 GE/µl	4/6	4/6	5/6	3/6	2/6
0,078 GE/µl	4/6	4/6	3/6	3/6	3/6

Was run by another laboratory technician.

* no template added (was missed)!

LOD is 1,25 GE/ul

Precision: Reproducibility

Concentration (GE/reaction)	Average (Cq)	Standard deviation (SD)	Variation cofficient (CV)
5	35.461	0.355	1,001
2,5	36.433	0.619	1,69
1,25	38.323	0.798	2,08

Run	Parameters
1-4	Variation over time: 2016-06-10 to 2016-06-15
5	Run by another laboratory technician 2016-06-16

Robustness

Risk analysis
This method is the best performed so far among all different 5 primer-probe combinations tested. It has 100% specificity for bv 1-4 but 89% if bv 5 is also taken into account. The method has good sensitivity. However, the validation evaluates only the performance of the method in analysis of pure DNA. When analyzing samples suspected to contain *B. suis*, the properties of the matrix must be taking into account. The matrix may for example inhibit the DNA polymerase. The evaluation should therefore be repeated in the presence of the relevant matrix.

1.5.3 Deviations

The method can’t detect *B. suis* bv 5.

1.5.4 Summary and conclusions

Summary

Performance	Result	Meets the requirements?
Specificity, inclusivity	89 %	Yes, partly
LOD	6,25 GE/reaction	Yes
Efficiency	113%	Yes, in theory
Robustness: The method is tested at different laboratories (SVA, FOHM) and by different laboratory workers with good results.		

Conclusion
In the exclusivity and the inclusivity tests the *B. suis* real-time PCR performed well on all the strains included in the panel. The PCR method detected *B. suis* bv 1-4 but unfortunately not bv 5.

1.6 **References**

Novel real-time PCR detection assay for Brucella suis. C. Hänsel, K. Mertens, M. C. Elschner, F. Melzer. Vet Rec Open 2015;2.

Projektrapport: harmonisering av odling och PCR detektion av Brucella. FBD 2014/14.

Kvalitetssäkring av realtids-PCR samt laboratoriesäkerhet för analys av högpatogena bakterier inom Forum för Beredskapsdiagnostik. FBD 2014/15.

KI-Discus Test

Enligt SS-EN 12469:2000

Kund: Ninolab AB
Kontrolldatum: 2014-02-27
Arbetsorder: 4070

Kontaktperson: Chris Ulric
Mättekniker: Jan Karlsson
Löpnummer: 351

Avdelning: SVA, Uppsala
Kontaktperson: Göran Holmstam
Lokal: F211:2

Utrustning: Scanlaf Mars 1200
Huvudfilter: 1st 1220x610x115
Drifttimmar: 1 041

Serienummer: L.07131883
Frånluftsfilter: 1st 457x610x69
Anläggning: -

Mätinstrument:
Modell: KI-Discus
Serienummer: K09/13-0274
Kalibreringsdatum: 2013-09-30
Kalibrerad till: 2014-09-30

Krav: För att uppnå en Skyddsfaktor på 10^5 får antal droplets inte överstiga 62 på något av filtrerna

Test		No of droplets counted	Graticule Multiplication factor (if approp)	Total droplets recovered (AxB=N)	Protection Factor $\frac{62 \times 10^5}{N}$	Pass / Fail
Top						
1	X	1	-	25	$2,48 \times 10^5$	Pass
	Y	1	-	10	$6,20 \times 10^5$	Pass
2	X	6	-	7	$8,86 \times 10^5$	Pass
	Y	14	-	10	$6,20 \times 10^5$	Pass
3	X	38	-	16	$3,87 \times 10^5$	Pass
	Y	17	-	13	$4,77 \times 10^5$	Pass
4	X	16	-	12	$3,88 \times 10^5$	Pass
	Y	10	-	9	$6,89 \times 10^5$	Pass
5	X	20	-	14	$4,43 \times 10^5$	Pass
	Y	9	-	12	$5,17 \times 10^5$	Pass
Bottom						
1	X	2	-	12	$5,17 \times 10^5$	Pass
	Y	1	-	5	$1,24 \times 10^6$	Pass
2	X	6	-	24	$2,58 \times 10^5$	Pass
	Y	6	-	25	$2,48 \times 10^5$	Pass
3	X	4	-	23	$2,70 \times 10^5$	Pass
	Y	2	-	10	$6,20 \times 10^5$	Pass
4	X	16	-	20	$3,10 \times 10^5$	Pass
	Y	10	-	13	$4,77 \times 10^5$	Pass
5	X	20	-	25	$2,48 \times 10^5$	Pass
	Y	10	-	10	$6,20 \times 10^5$	Pass

Vita Verita AB
Västra Rydsvägen 138
196 31 Kungsängen
Tel: 08-584 606 40 **Fax:** 08-584 606 49

Datum: 2014-02-28

Signatur:

CONTAINMENT
TECHNOLOGY

KI-DISCUS
CERTIFICATE OF TEST & CALIBRATION

Certificate No. CTQ 1627 Date of Issue: 30TH SEPTEMBER 2013

Test	Description	Target	Measured
1	Hyperdermic/Disc Clearance	0.1 mm (0.004 in)	0.1mm
2	Electronic Speed Controller Setting Note: Permitted Transients	28,000 RPM +/- 500 RPM	28,020 RPM
3	Vacuum Generated at Sensing Head	200 mm H_2O (8 inches)	200mmH_2O
4	Pump Delivery Potassium Iodide (Including run up time to pump start)	20 ml in 9 Mins. 15 Secs. +60 Secs / - 30 Secs.	9MINS. 03SECS

Instrument used to carry out Test 2.

Instrument : ISOTECH IGC 2231 Serial No : 9471034

Date of Calibration : 8TH MARCH 2013 Certificate No : 173201

KI-DISCUS Serial No: K09/13-0274

Tested by:-

Signature : [signature] Name : D. POLLINGTON

Calibration Date : 30TH SEPTEMBER 2013 Certificate No.: CTQ 1627

CTL FORM NO. CQ 0001
ISSUE E APRIL 2002

Containment Technology Limited
9 Telford Road, Ferndown Industrial Estate, Wimborne, Dorset BH21 7QW

KI-Discus Test

Enligt SS-EN 12469:2000

Kund: SVA | **Kontrolldatum:** 2014-02-27 | **Arbetsorder:** 6105

Kontaktperson: Olga | **Mättekniker:** Johan Byberger | **Löpnummer:** 351

Avdelning: BKT | **Lokal:** F211:2 | **Lokal:** F211:2

Utrustning: Scanlaf Mars 1200 | **Huvudfilter:** 1st 1220x610x115 | **Drifttimmar:** 9285

Serienummer: L.07131883 | **Frånluftsfilter:** 1st 457x610x69 | **Anläggning:** -

Mätinstrument:

Modell: KI-Discus | **Serienummer:** K09/13-0274

Kalibreringsdatum: 2015-10-30 | **Kalibrerad till:** 2016-10-30

Krav: För att uppnå en Skyddsfaktor på 10^5 får antal droplets inte överstiga 62 på något av filtrerna

Test		No of droplets counted	Graticule Multiplication factor (if approp)	Total droplets recovered (AxB=N)	Protection Factor $\frac{62 \times 10^5}{N}$	Pass / Fail
Top						
1	**X**	3	-	3	$2{,}07 \times 10^6$	Pass
	Y	5	-	5	$1{,}24 \times 10^6$	Pass
2	**X**	2	-	2	$3{,}10 \times 10^6$	Pass
	Y	6	-	6	$1{,}03 \times 10^6$	Pass
3	**X**	7	-	7	$8{,}85 \times 10^5$	Pass
	Y	5	-	5	$1{,}24 \times 10^6$	Pass
4	**X**	7	-	7	$8{,}85 \times 10^5$	Pass
	Y	4	-	4	$1{,}55 \times 10^6$	Pass
5	**X**	8	-	8	$7{,}75 \times 10^5$	Pass
	Y	6	-	6	$1{,}03 \times 10^6$	Pass
Bottom						
1	**X**	5	-	5	$1{,}24 \times 10^6$	Pass
	Y	10	-	10	$6{,}2 \times 10^5$	Pass
2	**X**	8	-	8	$7{,}75 \times 10^5$	Pass
	Y	27	-	27	$2{,}30 \times 10^5$	Pass
3	**X**	15	-	15	$4{,}13 \times 10^5$	Pass
	Y	5	-	5	$1{,}24 \times 10^6$	Pass
4	**X**	25	-	25	$2{,}48 \times 10^5$	Pass
	Y	10	-	10	$6{,}2 \times 10^5$	Pass
5	**X**	10	-	10	$6{,}2 \times 10^5$	Pass
	Y	8	-	8	$7{,}75 \times 10^5$	Pass

Vita Verita AB
Västra Rydsvägen 136
196 31 Kungsängen
Tel: 08-584 606 40 **Fax:** 08-584 606 49

Datum: 2016-04-07

Signatur: Jan Karlsson

Protocol for evaluation of extraction robots.

Materials

- *Bacillus cereus* strain F2085.
- *Francisella tularensis* Live Vaccine Strain (LVS)
- PBS + 0.02 % tween
- NaCl (0.9 %)
- BHI agar plates supplied with 0.1 % L-Cystein, 1 % Dextrose and 9 % horse blood for cultivation of *F. tularensis*.
- Blood agar plates for *B. cereus*.
- Sample matrices, see list protocol page 4
- Virioner till extraktionsrören.
- 50 mL centrifuge tubes.
- Cell density meter WPA CO8000
- Extraction kits: EZ1 DNA Tissue kit, MagDEA DX SV, innuPREP Stool DNA kit and innuPREP Bacteria DNA kit.
- Lysozyme and Proteinase K for the InnuPure samplepreparation
- Seal herpes virions, PhHV-1, internal positive control.
- PCR-reagents for detection of *Bacillus* and *Francisella.*
- Extraction robots: EZ1 Advanced, MagLEAD 6gC and InnuPure C16.
- Picodrop and Qubit to measure concentration and quality of DNA-extractions

Day 0 Cultivation of bacterial agents

Bacillus cereus and *Francisella tularensis* LVS are streaked onto agar plates.

Day 1

In a biosafety cabinet to avoid cross contamination: bacteria from agar plates are resuspended to OD_{600} = 1. *B. cereus* in PBS + 0.02 % tween and *F. tularensis* LVS in physiological NaCl (0.9 %).

F. tularensis LVS OD_{600} = 1, corresponds to approximately 6 × 10^9 CFU/mL.
Dilution series in NaCl (0.9 %):

I. 167 µL of 6 × 10^9 + 833 µL NaCl → 1 × 10^9
II. 100 µL of 1 × 10^9 + 900 µL NaCl → 1 × 10^8 CFU/mL
III. 100 µL of 1 × 10^8 + 900 µL NaCl → 1 × 10^7 CFU/mL
IV. 100 µL of 1 × 10^7 + 900 µL NaCl → 1 × 10^6 CFU/mL →used to inoculate samples.
V. 100 µL of 1 x 10^6 + 900 µL NaCl → 1 × 10^5 CFU/mL
VI. 100 µL of 1 x 10^5 + 900 µL NaCl → 1 × 10^4 CFU/mL
VII. 100 µL of 1 x 10^4 + 900 µL NaCl → 1 × 10^3 CFU/mL →used for viable count.
VIII. 100 µL of 1 x 10^3 + 900 µL NaCl → 1 × 10^2 CFU/mL

For viable count: plate 3 × 100 µL, of 1 × 10^2, 1 × 10^3 and 1 × 10^4. (**9 plates**, grow in 5 % CO_2 at 37 ˚C for 1-7 days, until visible colonies.)

B. cereus OD =1 corresponds to approximately 1.26 × 10^8 CFU/mL.
Dilution series in PBS + 0.02 % tween:

I. 793 µL of 1,26 × 10^8 + 207 µL PBS + tween → 1 × 10^8 CFU/mL
II. 100 µL of 1 × 10^8 + 900 µL PBS + tween → 1 × 10^7 CFU/mL
IX. 100 µL of 1 × 10^7 + 900 µL PBS + tween → 1 × 10^6 CFU/mL →used to inoculate samples.
III. 100 µL of 1 x 10^6 + 900 µL PBS + tween → 1 × 10^5 CFU/mL
IV. 100 µL of 1 x 10^5 + 900 µL PBS + tween → 1 × 10^4 CFU/mL
V. 100 µL of 1 x 10^4 + 900 µL PBS + tween → 1 × 10^3 CFU/mL →used for viable count.
VI. 100 µL of 1 x 10^3 + 900 µL PBS + tween → 1 × 10^2 CFU/mL

Viable count: plate 3 × 100 µL, of 1 × 10^2, 1 × 10^3 och 1 × 10^4. (**9 plates,** grow over night at 37 ˚C)

1. For the **solid sample types without enrichment**: Weigh 2 g of each matrix into a 50 mL centrifuge tube (1 per sample type) with 10 glass beads, add 18 mL BHI. Vortex for 2 minutes.
2. Wait > 2 minutes, for aerosols to settle.
3. From each tube: take 5 mL to a 15 mL tube for the inoculated sample. In a biosafety cabinet: Add 50 µL of *F. tularensis* LVS 1×10^6 CFU/mL and 50 µL of *B. cereus* 1×10^6 CFU/mL for a final concentration of approximately 10^4 cfu/mL for both these agents. Mix by vortexing thouroghly and let the tube rest at least 2 minutes before opened to let any aerosols settle.
4. For the uninoculated sample, take from the matrix + BHI in 50 mL tube.
5. For **EZ1** and **MagLEAD 6gC**: add 195 µL of each sample to tubes with 5 µL virions:
    a. Uninoculated sample: one tube from each extraction kit.
    b. Inoculated sample in duplicate: i.e. two tubes from each kit.
6. Load samples and consumables into **EZ1** and **MagLEAD 6gC** following the instructions shown on the instrument displays, with 100 µL elution volume.
7. For the **InnuPure C16** using **innuPREP Bacteria DNA Kit**:
    a. For liquid sample matrices (no addition of BHI): take 200 µL to a 1.5 mL tube and move on to (d), below. All other samples: Pellet 2 × 1 mL of the inoculated samples and 1 × 1 mL of uninoculated samples by centrifugation (1.5 mL tubes).
    b. Remove the supernatant by pipette, avoid disturbing the pellet.
    c. Resuspend the pellet in 200 µL TE buffer by gentle pipetting (to avoid aerosol).
    d. Add 15 µL Lysozyme vortex for 5 sec.
    e. Lyse at 37 °C until full lysis (clear sample) 15-30 minutes.
    f. Add 200 µL Lysis solution CBV and 20 µL Proteinase K. Vortex for 5 sec. To avoid cross contamination, only one tube should be open at a time.
    g. Incubate at 50 °C for 30 min.
    h. Add 5 µL seal herpes-virions to each sample.
    i. Load each sample (440 µL) into well 3 in reagent strip or plate.
    j. Extract with program Ext Lysis 200 C16_04, with 100 µL elution volume.
8. For the **InnuPure C16** using **innuPREP Stool DNA Kit**:
    a. Add 300 µL of liquid samples to 2 mL tubes and add 1 mL Lysis solution SLB. Dupicates for the inoculated samples and single tubes for the uninoculated samples.
    b. Resuspend/homogenise.
    c. Take 200 µL of the homogenate to a 1.5 mL screw cap tube (other tubes may spring open during heating at 95 ˚C, below).
    d. Add 200 µL Lysis solution SLB and 20 µL Proteinase K vortex for 5 sec.
    e. Incubate at 60 °C for 30 minutes in a thermomixer, 600 rpm.
    f. Incubate at 95 °C 10 minutes, thermomixer 600 rpm. **Cool tubes before they are opened,** otherwise the pressure from heating may cause splashes.
    g. Transfer the lysed samples to pre-filter in 2 mL collection tube.
    h. Centrifuge 8000 × g 1 minute. **Keep the tube with the filtrate, this is the sample.**
    i. Add 5 µL seal herpes-virions to each sample.
    j. Load 400 µL into well 3 in strip or plate, Ext Lysis 200 C16_04 elute 100 µL.

1. For **solid sample types with enrichment**: weigh 2 g matrix into 50 mL centrifuge tube with 10 glass beads, add 18 mL BHI, vortex 2 minutes.

2. Enrich at 37 °C over night (18-24 h).
3. **Continue day 2:** Shake tube 2 minutes, let rest for >2 minutes. Continue from #3 in protocol for solid sample types without enrichment, above.

1. For **liquid sample types** (no enrichment): portion 5 mL of each liquid sample type in a 15 mL tube for inoculation.
2. In a biosafety cabinet: Add 50 µL of *F. tularensis* LVS 1 × 10^6 CFU/mL and 50 µL of *B. cereus* 1 × 10^6 CFU/mL for a final concentration of approximately 10^4 cfu/mL for both these agents. Mix by vortexing thouroghly and let the tube rest at least 2 minutes before opened to let any aerosols settle.
3. For **EZ1** and **MagLEAD 6gC** add 195 µL of each sample to tubes with 5 µL virions:
    a. Uninoculated sample: take directly from liquid matrix to one tube for each kit type.
    b. Inoculated sample: take duplicates of the inoculated liquid matrices i.e. two tubes per sample type for each kit.
4. Load samples and consumables into **EZ1** and **MagLEAD 6gC** following the instructions shown on the instrument displays.

Matrices used for evaluation of extraction robots

Solid sample types, extraction before and after enrichment:

Chocolate powder
Rasberries
Spinach leaves
Minced meat

Sample types, solid and liquid, extracted without enrichment:

Baby food (fish)
Cream
Orange juice
Tap water
Wheat flour
Potting soil (store bought)
Soil (from outside)
Egg yolk
Blood
Feed
Spleen
Mosquitos
Swab
Faeces
Water, ground
Water, surface

EZ1 Advanced

Matrix	ng/ul	ng/ul	260/280	260/280
Chocolate powder	8.52	7.10	3.16	3.03
Chocolate powder, not spiked	8.07		2.71	
Rasberry	12.0	12.22	2.23	2.71
Rasberry, not spiked	11.76		2.72	
Spinach leaves	7.63	7.57	5.06	9.71
Spinach leaves, not spiked	7.62		3.82	
Minced meat	9.45	8.87	3.08	3.89
Minced meat, not spiked	9.40		4.40	
Baby food fish	7.92	7.65	4.45	6.34
Baby food, not spiked	7.67		3.50	
Cream	44.46	61.73	1.28	1.34
Cream, not spiked	39.29		1.39	
Orange juice	7.84	9.45	6.17	4.88
Orange juice, not spiked	8.17		3.31	
Chocolate powder, enriched	12.49	12.86	2.97	2.42
Chocolate powder, enriched, not spiked	13.53		2.85	
Rasberry, enriched	24.05	24.09	1.86	1.99
Rasberry, enriched, not spiked	22.80		1.80	
Spinach leaves, enriched	18.07	18.46	2.70	2.45
Spinach leaves, enriched, not spiked	17.48		2.56	
Minced meat, enriched	23.32	38.11	2.67	2.17
Minced meat, enriched, not spiked	29.63		2.35	
Tap water	6.65	6.90	8.64	6.37
Tap water, not spiked	7.61		4.08	
Wheat flour	21.63	25.56	2.23	2.26
Wheat flour, not spiked	24.94		2.10	
Potting soil	18.24	17.41	2.08	2.11
Potting soil, not spiked	24.07		1.68	
Soil, outdoor	21.47	52.12	1.87	1.54
Soil outdoor, not spiked	47.76		1.46	
Egg yolk	10.48	9.86	2.26	2.18
Egg yolk, not spiked	10.32		2.09	

PSS magLEAD 6gc

Matrix	ng/ul	ng/ul	260/280	260/280
Chocolate powder	45.27	47.58	1.21	1.18
Chocolate powder, not spiked	50.70		1.18	
Rasberry	47.26	45.24	1.41	1.43
Rasberry, not spiked	37.05		1.38	
Spinach leaves	4.45	3.46	2.19	1.61
Spinach leaves, not spiked	4.23		1.40	
Minced meat	39.10	40.66	2.03	2.01
Minced meat, not spiked	24.75		1.85	
Baby food fish	6.99	7.59	2.03	1.65
Baby food, not spiked	8.04		1.86	
Cream	13.18	7.68	1.31	1.31
Cream, not spiked	11.22		1.32	
Orange juice	15.01	8.34	1.63	1.68
Orange juice, not spiked	7.09		1.99	
Chocolate powder, enriched	53.72	54.19	1.55	1.60
Chocolate powder, enriched, not spiked	68.60		1.57	
Rasberry, enriched	38.80	37.86	1.70	1.83
Rasberry, enriched, not spiked	39.23		1.63	
Spinach leaves, enriched	43.56	47.31	1.97	2.04
Spinach leaves, enriched, not spiked	44.72		1.97	
Minced meat, enriched	57.85	68.62	2.04	2.03
Minced meat, enriched, not spiked	74.68		2.09	
Tap water	0.44	0.14	616.38	-0.24
Tap water, not spiked	0.12		2.01	
Wheat flour	92.08	99.82	1.89	1.87
Wheat flour, not spiked	85.57		1.94	
Potting soil	67.68	118.04	1.37	1.34
Potting soil, not spiked	76.93		1.35	
Soil, outdoor	56.38	67.61	1.33	1.33
Soil outdoor, not spiked	106.64		1.33	
Egg yolk	176.40	201.74	1.01	1.00
Egg yolk, not spiked	185.90		0.99	

Analytic Jena Innupure C16 Bact kit

Matrix	ng/ul	ng/ul	260/280	260/280
Chocolate powder	50.0	44.32	1.26	1.24
Chocolate powder, not spiked	40.92		1.19	
Rasberry	7.32	12.96	1.57	1.53
Rasberry, not spiked	13.49		1.36	
Spinach leaves	1.09	2.66	1.22	1.39
Spinach leaves, not spiked	1.56		1.09	
Minced meat	21.93	14.30	1.31	2.08
Minced meat, not spiked	14.59		1.93	
Baby food fish	1.15	1.13	-3.81	1.21
Baby food, not spiked	1.80		1.20	
Cream	3.24	1.79	1.34	1.41
Cream, not spiked	2.08		3.05	
Orange juice	27.24	24.80	1.74	1.81
Orange juice, not spiked	21.75		1.80	
Chocolate powder, enriched	63.90	52.27	1.64	1.52
Chocolate powder, enriched, not spiked	32.89		1.43	
Rasberry, enriched	32.63	61.57	1.78	1.92
Rasberry, enriched, not spiked	59.55		1.83	
Spinach leaves, enriched	71.75	66.59	2.10	2.06
Spinach leaves, enriched, not spiked	54.61		2.08	
Minced meat, enriched	95.48	89.63	2.11	2.06
Minced meat, enriched, not spiked	98.94		2.08	
Tap water	-0.08	0.53	0.08	-33.28
Tap water, not spiked	0.76		1.63	
Wheat flour	6.05	5.28	1.78	1.51
Wheat flour, not spiked	13.23		1.68	
Potting soil	98.98	84.84	1.36	1.40
Potting soil, not spiked	100.18		1.38	
Soil, outdoor	186.60	205.94	1.35	1.33
Soil outdoor, not spiked	186.17		1.34	
Egg yolk	114.24	121.28	1.21	1.22
Egg yolk, not spiked	95.79		1.18	

Analytic Jena Innupure C16 stool kit

Matrix	ng/ul	ng/ul	260/280	260/280
Chocolate powder	7.64	8.25	1.38	1.58
Chocolate powder, not spiked	7.35		1.42	
Rasberry	6.03	6.60	1.61	1.37
Rasberry, not spiked	7.09		1.20	
Spinach leaves	5.04	6.09	1.60	1.55
Spinach leaves, not spiked	4.70		1.42	
Minced meat	6.44	6.64	1.42	1.43
Minced meat, not spiked	6.96		1.50	
Baby food fish	4.64	6.10	1.49	1.67
Baby food, not spiked	5.06		1.57	
Cream	4.70	6.09	1.21	1.47
Cream, not spiked	4.21		1.56	
Orange juice	9.40	10.45	1.67	1.84
Orange juice, not spiked	10.99		1.64	
Chocolate powder, enriched	10.52	9.88	1.68	1.65
Chocolate powder, enriched, not spiked	7.18		1.55	
Rasberry, enriched	9.53	10.69	1.58	1.48
Rasberry, enriched, not spiked	9.75		1.59	
Spinach leaves, enriched	5.85	5.09	1.89	2.76
Spinach leaves, enriched, not spiked	3.95		1.71	
Minced meat, enriched	8.39	14.71	1.43	1.77
Minced meat, enriched, not spiked	10.80		1.69	
Tap water	4.15	10.89	2.19	1.93
Tap water, not spiked	3.80		1.59	
Wheat flour	3.56	7.70	2.10	1.82
Wheat flour, not spiked	5.19		1.80	
Potting soil	8.80	8.42	1.43	1.27
Potting soil, not spiked	9.29		1.43	
Soil, outdoor	6.52	7.72	1.36	1.89
Soil outdoor, not spiked	7.11		1.36	
Egg yolk	113.58	-3.03	1.20	1.83
Egg yolk, not spiked	2.24		1.69	

EZ1 Advanced

Matrix	ng/ul	260/280
Spleen 0	170,4 ng/µL	2,01
Spleen 1	175,7 ng/µL	2,03
Spleen 2	192,8 ng/µL	2,011
Excrement 0	10,1 ng/µL	1,571
Excrement 1	38,9 ng/µL	1,326
Excrement 2	42,2 ng/µL	1,307
Mosquito 0	1,9 ng/µL	4,307
Mosquito 1	7,3 ng/µL	2,139
Mosquito 2	5,4 ng/µL	1,503
Feed 0	8,8 ng/µL	1,871
Feed 1	8,8 ng/µL	1,693
Feed 2	5,6 ng/µL	1,95
WaterGround0	3,9 ng/µL	1,573
WaterGround1	3,4 ng/µL	1,38
WaterGround2	1,5 ng/µL	1,451
WaterSurface 0	0,8 ng/µL	1,422
WaterSurface 1	3,1 ng/µL	1,721
WaterSurface 2	0,9 ng/µL	1,381
Blood 0	21,2 ng/µL	1,755
Blood 1	25,7 ng/µL	1,852
Blood 2	23,2 ng/µL	1,979
Swab 0	91,4 ng/µL	2,053
Swab 1	64,6 ng/µL	2,027
Swab 2	65,9 ng/µL	1,998

PSS magLEAD 6gc

Matrix	ng/ul	260/280
Spleen 0	1259,5 ng/µL	1,777
Spleen 1	1283,1 ng/µL	1,742
Spleen 2	1292,3 ng/µL	1,701
Excrement 0	1,5 ng/µL	0,789
Excrement 1	168,4 ng/µL	1,805
Excrement 2	155,7 ng/µL	1,811
Mosquito 0	22,0 ng/µL	1,779
Mosquito 1	8,1 ng/µL	1,82
Mosquito 2	5,9 ng/µL	1,923
Feed 0	19,7 ng/µL	1,742
Feed 1	11,2 ng/µL	1,435
Feed 2	19,6 ng/µL	1,894
WaterGround0	0,2 ng/µL	-7,329
Water g 0	2,0 ng/µL	1,24
WaterGround1	-0,3 ng/µL	0,258
Water g 1	5,4 ng/µL	1,573
WaterGround2	0,6 ng/µL	-1,247
Water g 2	2,6 ng/µL	1,791
WaterSurface 0	-1,7 ng/µL	-1,252
Water s 0	-0,4 ng/µL	-2,078
WaterSurface 1	0,7 ng/µL	0,282
Water s 1	-1,5 ng/µL	-2,58
WaterSurface 2	-0,1 ng/µL	-0,129
Water s 2	0,7 ng/µL	0,313
Blood 0	44,3 ng/µL	1,882
Blood 1	40,8 ng/µL	1,791
Blood 2	43,8 ng/µL	1,909
Swab 0	97,7 ng/µL	2,11
Swab 1	71,2 ng/µL	2,048
Swab 2	97,6 ng/µL	2,066

Analytic Jena Innupure C16 Bact kit

Matrix	ng/ul	260/280
Bact Spleen 0	39,7 ng/µL	1,808
Bact Spleen 1	43,3 ng/µL	1,782
Bact Spleen 2	44,2 ng/µL	1,739
Bact Feed 0	51,1 ng/µL	1,865
Bact Feed 1	9,3 ng/µL	1,053
Bact Feed 2	2,2 ng/µL	0,346
Bact Blood 0	3,2 ng/µL	0,781
Bact Blood 1	-4,8 ng/µL	2,726
Bact Blood 2	0,0 ng/µL	-0,007
Bact Swab 0	13,3 ng/µL	3,875
Bact Swab 1	9,2 ng/µL	4,093
Bact Swab 2	10,5 ng/µL	3,837
Bact Mosquito 0	2,2 ng/µL	-0,975
Bact Mosquito 1	-0,1 ng/µL	0,045
Bact Mosquito 2	-2,3 ng/µL	0,382

Analytic Jena Innupure C16 stool kit

Matrix	ng/ul	260/280
Stool Excrement 0	12,1 ng/µL	1,847
Stool Excrement 1	10,9 ng/µL	1,585
Stool Excrement 2	13,3 ng/µL	1,561
Stool Feed 0	8,4 ng/µL	1,553
Stool Feed 1	9,9 ng/µL	1,626
Stool Feed 2	10,7 ng/µL	1,577
Stool WaterGround0	4,6 ng/µL	0,986
Stool WaterGround1	7,2 ng/µL	1,302
Stool WaterGround2	6,3 ng/µL	1,39
Stool WaterSurface 0	5,8 ng/µL	1,669
Stool WaterSurface 1	6,5 ng/µL	1,545
Stool WaterSurface 2	6,9 ng/µL	1,907
Stool Swab 0	15,1 ng/µL	1,877
Stool Swab 1	12,0 ng/µL	1,833
Stool Swab 2	15,6 ng/µL	1,573

Table 1. Matrices spiked with *Bacillus cereus* and analysed by real-time PCR targeting the rpoB-gene using PerfeCTa qPCR ToughMix.

	EZ1 Advanced			MagLEAD 6gC PSS			InnuPure C16 Jena bacteria kit			InnuPure C16 Jena Stool kit		
	Ct sample	Ct NEC	Ct IPC	Ct sample	Ct NEC	Ct IPC	Ct sample	Ct NEC	Ct IPC	Ct sample	Ct NEC	Ct IPC
Chocolate powder	ND	ND	31,1	ND	ND	31,3	ND	ND	32,6	ND	ND	32,6
Raspberries	ND	ND	32,1	38,1	ND	31,3	41,8	ND	34,2	ND	ND	32,6
Spinach leaves	42,7	ND	31,2	29,0	ND	31,0	28,9	ND	32,6	ND	ND	32,6
Minced meat	ND	ND	31,4	29,5	ND	30,8	28,6	ND	33,0	ND	ND	33,2
Baby food (fish)	36,0	ND	31,3	27,8	ND	30,7	31,2	ND	33,8	ND	ND	33,6
Cream	ND	ND	31,4	35,7	ND	31,3	37,0	ND	33,8	ND	ND	33,4
Orange juice	39,3	ND	31,4	ND	ND	35,9	34,8	ND	32,9	ND	ND	33,5
Chocolate powder, enriched	39,7	ND	31,7	43,2	ND	30,8	ND	ND	33,1	ND	23,6	33,0
Raspberries, enriched	ND	ND	31,6	37,8	ND	30,5	32,5	ND	32,6	22,2	23,1	32,1
Spinach leaves, enriched	18,4	18,9	31,1	17,3	17,3	30,3	17,3	17,6	32,4	ND	ND	32,9
Minced meat, enriched	18,4	18,2	31,6	16,8	16,5	30,1	16,7	16,7	32,8	ND	ND	32,5
Tap water	ND	ND	32,0	31,6	32,1	30,6	31,6	32,6	32,8	ND	ND	32,5
Wheat flour	33,8	ND	32,3	27,2	37,9	30,3	32,8	ND	36,5	23,2	23,2	32,9
Potting soil (store bought)	ND	ND	32,0	ND	ND	30,8	ND	ND	33,1	ND	ND	32,2
Soil (from outside)	ND	ND	30,0	ND	ND	29,9	ND	ND	ND	ND	ND	32,2
Egg yolk	ND	ND	ND	ND	ND	32,5	ND	ND	33,0	ND	ND	31,7
Blood	26,9	ND	32,3	30,6	ND	31,8	35,9	ND	34,6	-	-	-
Feed	29,9	ND	34,6	32,2	ND	38,0	34,1*	ND	35,3	ND	ND	32,4
Spleen	32,6	ND	33,0	ND	ND	34,8	28,9	ND	33,8	-	-	-
Mosquitos	26,3	ND	33,0	28,3	ND	34,2	28,2	ND	32,9	-	-	-
Swab	26,2	ND	33,3	31,0	ND	33,8	34,9	ND	33,4	ND	ND	32,7
Faeces	27,3	ND	33,9	31,3	ND	34,5	-	-	-	ND	ND	32,3
Water, ground	26,1	ND	33,3	30,0	ND	32,8	-	-	-	ND	ND	32,7
Water, surface	27,3	ND	33,2	30,7	ND	32,9	-	-	-	ND	ND	33,1

Table 2. Matrices spiked with *Francisella tularensis* and analysed by real-time PCR targeting the ISFtu2-gene using PerfeCTa qPCR ToughMix.

	EZ1 Advanced			MagLEAD 6gC PSS			InnuPure C16 Jena bacteria kit			InnuPure C16 Jena Stool kit		
	Ct sample	Ct NEC	Ct IPC	Ct sample	Ct NEC	Ct IPC	Ct sample	Ct NEC	Ct IPC	Ct sample	Ct NEC	Ct IPC
Chocolate powder	26,4	ND	31,4	26,5	ND	31,4	30,7	ND	32,7	30,1	ND	32,9
Raspberries	27,1	ND	32,3	25,8	ND	31,4	29,7	ND	34,0	30,8	ND	32,6
Spinach leaves	26,1	ND	31,4	25,3	ND	31,1	26,6	ND	32,5	30,0	ND	32,7
Minced meat	26,4	ND	31,6	25,1	ND	30,8	26,3	ND	32,9	30,6	ND	32,9
Baby food (fish)	25,9	ND	31,4	25,7	ND	30,7	28,2	ND	33,8	31,4	ND	33,5
Cream	32,1	ND	31,8	25,7	ND	31,8	29,9	ND	35,1	33,3	ND	33,6
Orange juice	26,6	ND	31,5	30,5	ND	36,0	29,0	ND	32,9	31,2	ND	33,4
Chocolate powder, enriched	26,3	ND	31,3	26,4	ND	30,4	29,2	ND	32,6	31,4	31,88*	32,7
Raspberries, enriched	26,0	ND	30,8	25,0	ND	30,1	25,4	39,63*	32,1	30,6	ND	31,8
Spinach leaves, enriched	25,9	ND	30,7	25,8	ND	30,1	25,8	ND	32,1	32,0	ND	32,5
Minced meat, enriched	26,0	ND	31,1	24,8	ND	30,0	26,0	ND	32,4	35,3	ND	32,0
Tap water	27,1	ND	31,7	25,7	ND	30,1	28,2	37,56*	32,5	30,5	ND	32,1
Wheat flour	26,7	36,61*	32,0	25,8	ND	30,0	31,2	ND	37,0	31,3	ND	32,3
Potting soil (store bought)	26,0	ND	31,6	27,0	ND	30,8	ND	ND	32,9	29,7	ND	32,1
Soil (from outside)	27,5	ND	30,0	32,5	ND	29,9	ND	ND	ND	30,2	ND	32,3
Egg yolk	36,4	ND	ND	29,8	ND	32,5	28,2	ND	33,2	29,2	ND	31,7
Blood	23,4	ND	31,0	22,2	ND	31,1	27,1	34,9*	35,2	-	-	-
Feed	24,9	ND	33,8	27,9	ND	34,4	34,2	ND	33,5*	29,5	ND	32,1
Spleen	24,2	ND	31,8	ND	ND	35,3	25,9	ND	33,5	-	-	-
Mosquitos	23,1	ND	31,9	22,2	ND	33,1	24,6	ND	32,8	-	-	-
Swab	23,1	ND	33,1	22,5	ND	33,5	26,2	ND	33,4	29,2	ND	32,2
Faeces	22,9	ND	32,8	22,9	ND	33,6	-	-	-	28,4	ND	31,8
Water, ground	22,7	ND	32,1	22,1	ND	32,0	-	-	-	30,6	ND	32,4
Water, surface	23,3	ND	31,8	22,2	ND	32,2	-	-	-	30,1	ND	33,0

Table 3. Matrices spiked with *Bacillus cereus* and analysed by real-time PCR targeting the rpoB-gene using Immolase DNA Polymerase.

	EZ1 Advanced			**MagLEAD 6gC PSS**			**InnuPure C16 Jena bacteria kit**			**InnuPure C16 Jena Stool kit**		
	Ct sample	Ct NEC	Ct IPC	Ct sample	Ct NEC	Ct IPC	Ct sample	Ct NEC	Ct IPC	Ct sample	Ct NEC	Ct IPC
Chocolate powder	37,1	ND	30,3	ND	ND	30,5	ND	ND	31,4	ND	ND	32,0
Raspberries	ND	ND	31,1	ND	ND	30,5	ND	ND	33,1	ND	ND	31,1
Spinach leaves	37,2	ND	30,6	31,3	ND	30,3	34,7	ND	31,7	ND	ND	31,4
Minced meat	ND	ND	30,9	33,6	ND	30,0	32,6	ND	31,9	ND	ND	32,3
Baby food (fish)	39,87**	ND	30,4	31,1	ND	30,3	ND	ND	31,9	ND	ND	32,0
Cream	ND	ND	ND	ND	ND	30,3	42,9	ND***	33,7	ND	ND	32,0
Orange juice	37,2	ND	31,0	ND	ND	35,1	ND	ND	31,1	20,1	ND	28,5
Chocolate powder, enriched	28,9	39,8	32,0	29,2	ND	31,2	28,8	35,5	32,1	28,9	19,3	33,0
Raspberries, enriched	30,4	39,6	31,3	28,6	ND	30,0	26,7	36,9	32,2	14,2	19,7	32,0
Spinach leaves, enriched	15,1	15,3	31,9	15,4	14,5	30,0	14,2	14,1	32,0	36,2	35,7	32,7
Minced meat, enriched	15,4	15,5	31,6	15,0	14,3	29,9	13,9	14,3	32,3	31,2	39,2	31,5
Tap water	34,4	ND	32,6	29,7	30,2	30,8	29,5	29,1	33,5	36,3	ND	33,5
Wheat flour	30,5	35,7	32,5	22,0	26,0	29,9	24,7	25,8	35,5	27,6	20,8	32,6
Potting soil (store bought)	36,5	ND	31,4	34,7	ND	30,9	ND	ND	39,3	29,1	ND	32,0
Soil (from outside)	25,3	ND	30,2	40,2	ND	30,3	ND	ND	ND	14,5	ND	24,1
Egg yolk	ND	ND	ND	39,3	ND	28,2	33,2	ND	32,2	33,1	ND	31,3

Table 4. Matrices spiked with *Bacillus cereus* and analysed by real-time PCR targeting the rpoB-gene using PerfeCTa MultiPlex qPCR SuperMix.

	EZ1 Advanced			MagLEAD 6gC PSS			InnuPure C16 Jena bacteria kit			InnuPure C16 Jena Stool kit		
	Ct sample	Ct NEC	Ct IPC	Ct sample	Ct NEC	Ct IPC	Ct sample	Ct NEC	Ct IPC	Ct sample	Ct NEC	Ct IPC
Chocolate powder	31,6	ND	30,6	29,5	ND	30,0	31,8	ND	31,7	35,9	ND	31,5
Raspberries	31,0	ND	30,9	ND	ND	ND	32,4	ND	34,9	34,7	ND	32,1
Spinach leaves	31,9	36,1	30,5	30,8	37,0	30,5	31,9	36,1	31,8	34,8	40,0	31,8
Minced meat	33,0	ND	31,6	26,9	ND	30,1	25,5	ND	32,1	35,2	ND	32,0
Baby food (fish)	31,0	ND	30,8	25,7	37,2	30,1	27,5	ND	32,8	34,0	ND	32,6
Cream	33,3	ND	32,2	ND	ND	34,0	34,2	ND	33,5	34,9	ND	32,5
Orange juice	31,1	ND	31,1	36,8	ND	34,8	29,4	ND	31,7	34,6	ND	32,9
Chocolate powder, enriched	30,8	ND	31,6	29,5	ND	30,1	28,8	ND	32,2	34,0	20,8	32,0
Raspberries, enriched	ND	ND	34,2	ND	ND	41,9	ND	ND	31,5	20,2	20,4	31,5
Spinach leaves, enriched	16,4	16,5	30,8	16,1	16,1	29,5	15,4	15,3	31,5	ND	ND	32,0
Minced meat, enriched	16,3	16,1	30,5	15,8	15,7	29,2	15,0	15,0	32,1	ND	37,9	31,5
Tap water	34,7	ND	31,8	30,1	30,4¤	30,0	29,3	29,7*	32,0	36,7	ND	32,0
Wheat flour	30,6	ND	32,0	22,7	30,4	29,9	30,8	ND	35,4	29,5**	21,1	31,6
Potting soil (store bought)	ND	ND	33,3	ND	ND	ND	ND	ND	ND	ND	ND	31,0
Soil (from outside)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	30,3
Egg yolk	ND	ND	ND	30,5	ND	30,6	31,8	ND	32,5	34,8	ND	30,3

Green colour indicates that detection is ok (for the NEC this is usually the same as no detection if the detection of IPC is also ok)..

Yellow colour indicates that the detection is in some way impaired, e.g high Ct value for one or more replicates. A high Ct value can be caused by inhibition or if the extraction has not retrieved the target DNA efficiently from the sample.

Red colour indicates that there is no detection of target genes in the inoculated sample, or that the IPC is not detected in the NEC.

* Results from only one of the replicates

** Large spread of the two replicates, mean value ok

*** Curve shows no value although Ct = 19,5

¤ Water giving a strong signal for bacillus, possible contamination?

Values marked with dotted lines are likely due to cross contamination.

ND = not detected

NEC = negative extraction control, uninoculated sample

IPC = internal process control

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