令和元年度厚生労働行政推進調査事業費補助金
（新興・再興感染症及び予防接種政策推進研究事業）

我が国で開発され，備蓄されている細胞培養痘そうワクチンの有効性，安全性，生産性向上および国内外のバイオテロ対策のあり方に関する研究

分担報告書

細胞培養弱毒生痘そうワクチンの特性解析（遺伝子機能解析），品質試験法に関する研究

所　属　　国立感染症研究所・獣医科学部・部長
研究分担者　　前田　健

研究要旨：2019 年 7 月に Global Health Security Action Group-Laboratory Network（GHSAG-LN）よりバイオテロに関する細菌系 5 種，炭疽菌・ペスト菌・ブルセラ菌・野兎病菌・類鼻疽菌の検査の外部精度管理(EQA)の実施が提案された．GHSAG-LN の日本の責任者であり，かつ，本研究班の研究代表者でもある西條博士の指導のもと，国立感染症研究所の細菌第一部，細菌第二部，獣医科学部の 3 部で対応した．生菌パネルと DNA パネルの選択が提示されたが，日本としては DNA パネルでの検査対応を行った．その結果を 2 月 14 日に報告した．

研究協力者：奥谷晶子，今岡浩一，堀田明豊（同，獣医科学部），石原智子，川端寛樹，大西　真（同，細菌一部），堀田敦子，柴山恵吾（同，細菌二部），西條政幸（同，ウイルス第一部）

A. 研究目的

世界健康安全保障イニシアティブ(Global Health Security Initiative：GHSI)には G7（カナダ，フランス，ドイツ，イタリア，日本，英国，米国），メキシコ，欧州委員会（EC）で構成され，オブザーバーとして WHO も参加している．世界健康安全保障行動グループ（GHSAG）は各国の局長級実務者で構成され，大臣らの計画と目的を具体的な行動に移し，危機が発生した際に迅速なコミュニケーションのネットワークとして機能することを目的としている．GHSAG のメンバーは，地球規模の健康安全保障の問題について情報を交換し，GHSI ネットワークの政策の優先事項を協議し，技術レベルでの行動の進捗状況を確認し，閣僚級会合の準備を支援する．

B. 研究方法

GHSAG ラボネットワークでは議長はカナダとメキシコ，診断の質の保証，診断手法・技術の柔軟性と適応性の向上や，検体の輸送の問題に取り組んでいる．

2019 年 7 月に議長国のカナダより，炭疽菌，野兎病菌，ペスト菌，ブルセラ属菌，バークホルデリア属菌の EQA が実施された．

カナダより資料 1 のように生菌のパネルで検査するか，DNA のパネルで検査のどちらかを選択するように依頼が来た．関係者での討議の後，日本では DNA パネルで検査を実施することを決定した．

【倫理面への配慮】

該当しない．

C. 研究結果

添付資料 2 のように 2020 年 1 月 27 日に菌由来の DNA がスキムミルク，グリセロールの入った DNA が 5 種類送られてきた．中身は不明であった．5 日以内に結果を出すようにということなので，2 月 10 日検査開始，2 月 14 日を報告期限として検査を実施した．

1 日目は DNA の抽出精製を試みた．QIAamp DNA blood Mini Kit を用いて DNA を精製後，精製 DNA を各菌の DNA 検査のために 40μL ずつ送付した．

2 日目以降は各菌の担当者により検査が実施された．ブルセラ菌の検査は資料 3，ペスト菌の検査は資料 4，バークホルデリアの検査は資料 5，野兎病菌の検査は資料 6，炭疽菌の検査は資料 7 にまとめられている．2 月 13 日に最終結果として，15A Bacillus spp，15B Bacillus anthracis，15C Bacillus anthracis and the other bacteria，15D No bacteria，15E Bacillus anthracis と報告した．

D. 考察

国立感染症研究所の 3 つの部が協力して，EQA に対応した．結果としては，Bacillus 属の

DNA しか存在していなかったが, 5 種のバイオテロの対象となる各種病原体に対して専門的に行っている各部が協調し, 迅速・適切に対応できた.

E. 結論

国内で細菌系のバイオテロが発生した場合も迅速かつ適切に同定できることが確認された. 今後も, DNA ウイルス, RNA ウイルスなどによるバイオテロの発生した際の対応なども併せて必要だと考えられた.

F. 健康危険情報

特になし

G. 研究発表

1. 論文発表

なし

2. 学会発表

なし

H. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

資料 1

National Microbiology Laboratory Biothreat Panel Request Survey for the GHSAG Laboratory Network

The NML can offer a variety of security sensitive biological agent (SSBAs) or near neighbors to determine success in identification. These panels would focus only on bacterial agents of security concern and may be provided as non-viable molecular panels. They would be available to labs that have CL3 capability, and preferably those laboratories that have experience in the triage and identification of unknowns for biosecurity investigations. These panels can be offered as live agent panels; however interest and importation requirements will further determine the panel design.

Instructions for use of this table are on page 2.

Country	B. anthracis, F. tularensis, Y. pestis, Brucella spp. Burkholderia spp.		Recipient Contact Name and Institutional Mailing Address	Recipient Telephone Fax and Email
	Live	Dead		
FRANCE	☒	☒	Anne LE FLECHE Cellule d'Intervention Biologique d'Urgence INSTITUT PASTEUR 25-28 rue du Dr Roux 75015 Paris	T: +33 1 40 61 38 08 F: +33 1 40 61 38 07 E: anne.le-fleche@pasteur.fr
GERMANY	☒	☒	Prof Dr Roland Grunow Robert Koch Institute, ZBS 2 Seestr. 10 13353 Berlin, Germany	T: +49 30 18754 2100 F: +49 30 18754 2110 E: GrunowR@rki.de
ITALY	☒ Y. pestis only	☒	Dr. Antonino Di Caro Director - Microbiology Laboratory National Institute for Infectious Diseases - Lazzaro Spallanzani Via Portuense, 292 00149 Rome, Italy	T:+390655170685 F:+390655170683 E: antonino.dicaro@inmi.it
JAPAN	☐	☒	Dr. Ken Maeda Director – Department of Veterinary Medical Science National Institute of Infectious Diseases 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan	T: +81-3-4582-2750 F: +81-3-5285-1179 E: kmaeda@nih.go.jp
MEXICO	☐	☐		T: F: E:
UNITED KINGDOM	☐	☐		T: F: E:
UNITED STATES	☐	☐		T: F: E:

Instructions on the use of this table.

1. Download the survey onto your computer.
2. Select the types of samples you wish to receive by clicking on the boxes.
3. Fill in the details for the appropriate contact person/recipient, institutional mailing address, and contact information for the recipient.
4. Save your survey and email it back to me. I will collate everyone's preferences.

資料 2

Government of Canada / Gouvernement du Canada
Public Health Agency of Canada / Agence de santé publique du Canada

CLRN RCLI

Canadian Laboratory Response Network Annual Proficiency

This proficiency panel will include several elements, including testing and analysis components to identify security sensitive biological agents, as well as the correct and timely reporting of results.

Five (5) samples will be provided to your laboratory from the National Microbiology Laboratory, Public Health Agency of Canada.

Please find attached the 'Acknowledgement of Receipt' Form. This must be faxed or emailed to the Bioforensics Assay Development and Diagnostics (BADD) laboratory once the samples are received.

Online reporting must be completed via the Canadian Laboratory Response Network (CLRN) Collaboration Site using the Canadian Network for Public Health Intelligence (CNPHI) platform. The reporting instructions are included in this package. You must report your results within 5 **calendar days from the start of the testing**; not from the package receipt.

Your laboratory will conduct testing on the provided nucleic acid material for the detection and identification of any of the following bacteria using your applicable procedures:

- *Bacillus anthracis*
- *Yersinia pestis*
- *Francisella tularensis*
- *Brucella sp*
- *Burkholderia pseudomallei*

If you have any questions, please contact the CLRN Office.

Good Luck!

Contact info: Bioforensics Assay Development and Diagnostics Laboratory
Email: phac.nml.badd-eedmm.lnm.aspc@canada.ca
Fax: (204) 789-5009

Canadian Science Centre for Human and Animal Health
1015 Arlington Street
Winnipeg, MB CANADA R3E 3R2

Instructions for Analysis and Reporting

Upon Receipt of Shipment: Upon arrival, document whether the outer box is intact and in good condition, and record the temperature within the box. Immediately notify the NML of package receipt by faxing or emailing a PDF of the enclosed Acknowledgement of Receipt Form to 1-204-789-5009 or phac.nml.badd-eedmm.lnm.aspc@canada.ca.

The outer packaging, as well as the data logger is the property of the courier and will be taken back immediately by a World Courier representative. Verify the inner box and security seal is intact. Contact the BADD laboratory in case of damage or a broken seal.

Contents: Package contains 5 DNA extracts in skim milk, glycerol and water. Each tube contains 500 uL.

Viability Disclaimer: The proficiency provider follows stringent Containment Level 3 standard operating procedures to assess the viability of hazardous biological material prior to removal from containment. The material provided herein has been extracted using a MasterPure™ DNA Purification Kit (Epicentre-Illumina), passed through a 0.22uM filter and 10% plated for growth. No growth was recovered following these practices but the use of stringent biosafety practices are recommended.

Instructions/Storage: Samples will arrive on dry ice. The samples must be stored at -20°C upon receipt to ensure stability of proficiency material provided.

Confidentiality: This proficiency panel must be completed without consultation with other laboratories.

Testing Instructions: You are receiving samples that mimic a submission requiring microbiological analysis from local law enforcement. The samples have undergone chemical, radiological and explosives screening and should be treated as microbiological samples. Samples are to be tested using processes that are applicable to your facility. As in a real event, timely reporting is critical. Your laboratory must report your preliminary results within 5 **calendar days** from the **start of the testing**. Final results are due March 2, 2020.

Reporting: The results will be reported as per "CNPHI CLRN Proficiency Test Database Access and Reporting Instructions (BADD-WI-007)" which is included in this package. This must be completed within the specified time period.

Sample Destruction: Contents of the package and all remaining samples must be destroyed upon completion of proficiency testing. Please find attached the 'Confirmation of Destruction' Form. This must be faxed or emailed to the Bioforensics Assay Development and Diagnostics (BADD) laboratory once the samples are destroyed.

Appeal Process: If you do not agree with the proficiency testing evaluation, you may appeal the decision by contacting phac.nml.badd-eedmm.lnm.aspc@canada.ca.

Government of Canada / Gouvernement du Canada
Public Health Agency of Canada / Agence de santé publique du Canada

CLRN RCLI

CLRN PROFICIENCY PANEL
CLRN_PT_2020

ACKNOWLEDGEMENT OF RECEIPT OF PROFICIENCY TEST SAMPLES

FAX (204) 789-5009 or email scanned copy to phac.nml.badd-eedmm.lnm.aspc@canada.ca

I Ken Maeda, National Institute of Infectious Diseases
(Laboratory Coordinator / Personnel) (Institution / Agency)

1-23-1 Toyama, Shinjuk-u, Tokyo, +81-3-4582-2750
(Address) (Telephone)

have received the **CLRN_PT_2020** proficiency test samples from National Microbiology Laboratory, Public Health Agency of Canada, located at the Canadian Science Centre for Human and Animal Health in Winnipeg, Manitoba.

- ☑ The outer box of the package is intact and the temperature inside the box has been verified. The temperature and time of the shipment upon delivery was appropriate on Jan. 27, 2020.
- ☑ The outer packing and data logger have been returned to the World Courier representative.
- ☑ Test samples will be destroyed upon completion of panel testing.
- ☑ This panel will be run according to the specified procedures of the facility and without consultation with other laboratories.

By signing this document, I confirm that I have received the correct panel that is appropriate for the capabilities within my facility.

(Signature)

Jan 29, 2020
(Date and Time)

資料 3

Detection of Brucella-Specific Gene

Dr. Koichi IMAOKA
Laboratory Chief
Laboratory of Reservoir Control of Zoonoses
Department of Veterinary Science
National Institute of Infectious Diseases

Outline:

A combinatorial PCR procedure identifies four major species of the genus *Brucella* (*B. melitensis, B. abortus, B. suis and B. canis*), simultaneously. The four pairs of primers targeting the genes encoding a cell surface protein (*BCSP31*) and outer membrane proteins (*omp2b*, *omp2a* and *omp31*) are prepared. PCR using these primers gives rise to specific patterns of amplification for each *Brucella* spp. (Imaoka, K. et.al., Simultaneous detection of the genus Brucella by combinatorial PCR. Jpn. J. Inf. Dis., 60:137-139, 2007).

Primer pairs:

1. A pair of primers **B4/B5** amplifies a 224-bp DNA fragment from the gene encoding a 31-kDa cell surface protein (BCSP31), which is well conserved in all *Brucella* spp. (M20404).
2. Two antisense primers, JPR-ab and JPR-ca, which are specific for *B. abortus* (U26438) and *B. canis* (U26439), respectively, are prepared.
   A pair of primers **JPF/JPR-ab** amplifies a 186-bp fragment from *B. abortus*, *B. melitensis* and *B. suis* but not from *B. canis*.
   A pair of primers **JPF/JPR-ca** amplifies a 187-bp fragment from *B. canis* and *B. suis*.
   *(The gene encoding Brucella major outer membrane protein 2 (omp2) has two related regions, omp2b and omp2a, and these two regions are 85% homologous and oriented in opposite directions (U26438).)
3. A pair of primers **1S/1AS** amplifies a 249-bp fragment from the omp31 gene encoding Brucella outer membrane protein of *B. melitensis*, *B. suis* and *B. canis* but not from *B. abortus* (AF366073), because of the presence of a large deletion in the omp31 gene of *B. abortus*.

Fig) Summary of PCR target gene, Primer pair, Product size and Brucella strains which show positive amplification

Target gene		Primer pair	Product size	Positive
bcsp31		B4/B5	224 bp	BM, BA, BS, BC
omp2	(abortus type)	JPF/JPR-ab	186 bp	BM, BA, BS
	(canis type)	JPF/JPR-ca	187 bp	BS, BC
omp31		1S/1AS	249 bp	BM, BS, BC

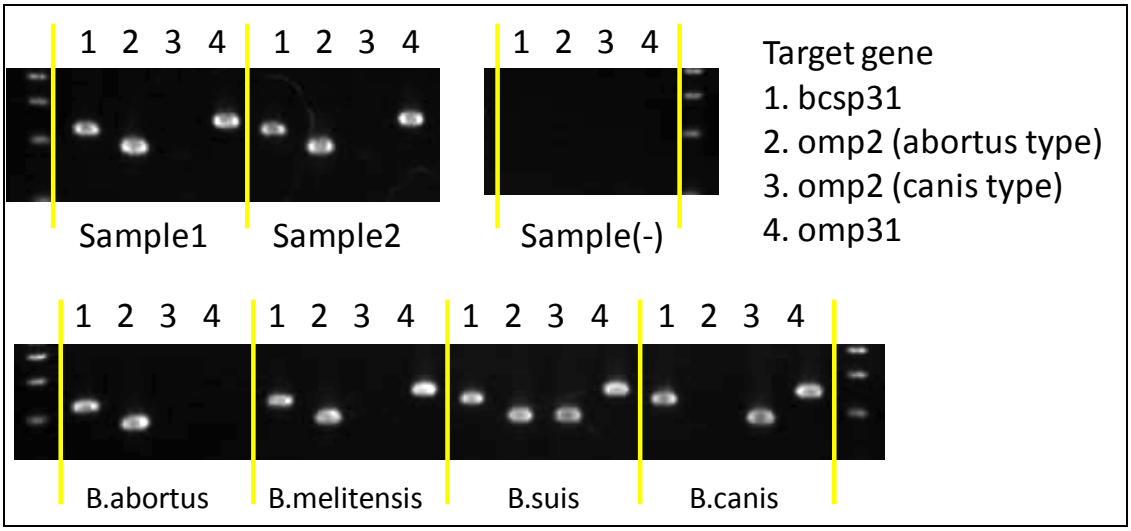
BM: *Brucella melitensis*, BA: *B. abortus*, BS: *B. suis*, BC: *B. canis*

Fig) Primers designated for a combinatorial PCR

Target gene	Primer name	Sequence
BCSP31	B4 (S)	5'-Tgg CTC ggT TgC CAA TAT CAA
	B5 (AS)	5'-CgC gCT TgC CTT TCA ggT CTg
omp2	JPF (S)	5'-gCg CTC Agg CTg CCg ACg CAA
	JPR-ab (AS)	5'-CAT TgC ggT Cgg TAC Cgg Ag
	JPR-ca (AS)	5'-CCT TTA CgA TCC gAg CCg gTA
omp31	1S (S)	5'-gTT CgC TCg ACg TAA CAg CTg
	1AS (AS)	5'-gAC CgC Cgg TAC CAT AAA CCA

Example: Amplification patterns of a combinatorial PCR

(Sample 1&2 are identified as *B. melitensis* from an amplilfication pattern)



Strains	Positive amplification (**primer pair**)
B. abortus	bcsp31 (**B4/B5**), omp2 (abortus-type)(**JPR/JPF-ab**)
B. melitensis	bcsp31 (**B4/B5**), omp2 (abortus-type)(**JPR/JPF-ab**), omp31 (**1S/1AS**)
B. suis	All: bcsp31 (**B4/B5**), omp2 (abortus-type)(**JPR/JPF-ab**), omp2 (canis-type)(**JPR/JPF-ca**), omp31 (**1S/1AS**)
B. canis	bcsp31 (**B4/B5**), omp2 (canis-type)(**JPR/JPF-ca**), omp31 (**1S/1AS**)

Work sheet for a combinatorial PCR for Brucella gene detection

Day1:

1. Profiles of Samples

#1-5 (A-E): Purified DNA samples from each test tubes were used as test samples.
#6: Purified DNA from *Brucella suis* strain 1330 was used as a positive control.
#7: D2W was used as a negative control.

Reaction tube #	1	2	3	4	5	6	7
(Samples)	A	B	C	D	E	PC (BS)	NC (D2W)
DNA conc. (ng/ul)	1.9	2.1	2.5	1.9	1.8	1.0	-

2. PCR

1) Preparation of puReTaq Ready-To-Go PCR Beads (Start at 9:00)
（RTG PCR Beads： #27-9559-01： GE Healthcare，2~2.5unit puReTaq DNA polymerase, 10mM Tris-HCl pH9.0, 50mM KCl, 1.5mM MgCl2, 200uM dNTP, BSA）
(1) Prepare 24 tubes of puRe Taq RTG PCR Beads
(2) Mark lids of tubes as b1~b7, a1~a7, c1~c7, o1~o7

2) Preparation of PCR reaction mixture
Prepare volume of each reaction mixture for 8 samples

b) For *bcsp31* detection (B4 & B5)

Reagents	1 reaction	8 reaction
D2W	20.5 µl	164 µl
Forward primer: B4 (10µM)	1 µl (0.4µM)	8 µl
Reverse primer: B5 (10µM)	1 µl (0.4µM)	8 µl
Total	22.5 µl	180 µl

Mix reagents in 1.5ml microtube and spin down by a centrifuge
Then, add 22.5ul of reaction mixture to b1~b7 tubes of puRe Taq RTG Beads tubes

a) For *omp2* abortus-type detection (JPF & JPR-ab)

Reagents	1 reaction	8 reaction
D2W	20.5 µl	164 µl
Forward primer: JPF (10µM)	1 µl (0.4µM)	8 µl
Reverse primer: JPR-ab (10µM)	1 µl (0.4µM)	8 µl
Total	22.5 µl	180 µl

Mix reagents in 1.5ml microtube and spin down by a centrifuge
Then, add 22.5ul of reaction mixture to a1~a7 tubes of puRe Taq RTG Beads tubes

c) For *omp2* canis-type detection (JPF & JPR-ca)

Reagents	1 reaction	8 reaction
D2W	20.5 µl	164 µl
Forward primer: JPF (10µM)	1 µl (0.4µM)	8 µl
Reverse primer: JPR-ca (10µM)	1 µl (0.4µM)	8 µl
Total	22.5 µl	180 µl

Mix reagents in 1.5ml microtube and spin down by a centrifuge
Then, add 22.5ul of reaction mixture to c1~c7 tubes of puRe Taq RTG Beads tubes

0) For *omp31* detection (1S & 1AS)

Reagents	1 reaction	8 reaction
D2W	20.5 µl	164 µl
Forward primer: B4 (10uM)	1 µl (0.4uM)	8 µl
Reverse primer: B5 (10uM)	1 µl (0.4uM)	8 µl

Total	22.5 ul	180 ul

Mix reagents in 1.5ml microtube and spin down by a centrifuge
Then, add 22.5ul of reaction mixture to o1~o7 tubes of puRe Taq RTG Beads tubes

3) Enter samples to each tube

(1) At first, add 2.5ul of Sample 7 (NC: D2W) to b7, a7, c7 and o7 tubes and close lids.
(2) Then, add 2.5ul of Sample 1 (A) to b1, a1, c1 and o1 tubes and close lids.
Add 2.5ul of Sample 2 (B) to b2, a2, c2 and o2 tubes and close lids.
Add 2.5ul of Sample 3 (C) to b3, a3, c3 and o3 tubes and close lids.
Add 2.5ul of Sample 4 (D) to b4, a4, c4 and o4 tubes and close lids.
Add 2.5ul of Sample 5 (E) to b5, a5, c5 and o5 tubes and close lids.
(3) Finally, add 2.5ul of Sample 6 (PC: BS) to b6, a6, c6 and o6 tubes and close lids.
(4) Gently centrifuge each tube

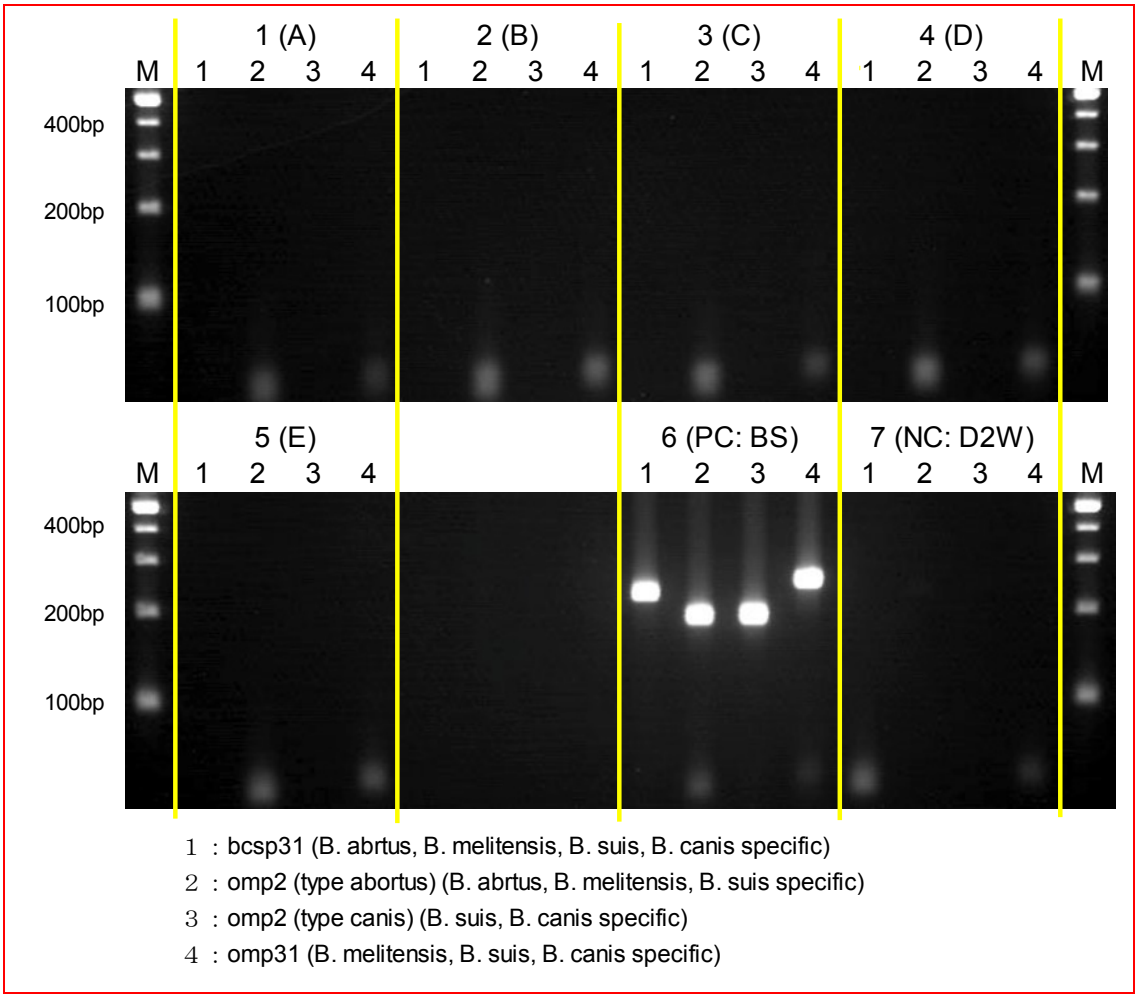
4) PCR

Set tubes to the thermal cycler (ABI: GeneAmp PCR System 9700)

Programs: 95 °C, 5 min
⇒ x 35 cycles (95 °C, 1 min → 65 °C, 1 min → 72 °C, 1 min)
⇒ 72 °C, 7 min ⇒ 4 °C

Start at 9:30
and Stop PCR at 12:30

5) Electrophoresis
Start at 13:00 and Stop electrophoresis at 13:50



1 : bcsp31 (B. abrtus, B. melitensis, B. suis, B. canis specific)
2 : omp2 (type abortus) (B. abrtus, B. melitensis, B. suis specific)
3 : omp2 (type canis) (B. suis, B. canis specific)
4 : omp31 (B. melitensis, B. suis, B. canis specific)

3. Results

Reaction tube #		Amplicons				Identified Brucella strains
		bcsp31 (224bp)	*omp2-ab* (186bp)	*omp2-ca* (187bp)	*omp31* (249bp)	
1	A	−	−	−	−	Negative
2	B	−	−	−	−	Negative
3	C	−	−	−	−	Negative
4	D	−	−	−	−	Negative
5	E	−	−	−	−	Negative
6	PC (BS)	+	+	+	+	B. suis
7	NC (D2W)	−	−	−	−	Negative

4. Conclusion

Brucella-specific gene were not detected in any samples from each test tubes (A-E).
Samples A-E did not include Brucella spp.

資料 4

Preparation of reaction mixture (for 10 reactions)

10X Ex Taq Buffer	25 ml
dNTP Mixture (2.5 mM each)	20 ml
10 mM caf1-F	25 ml
10 mM caf1-R	25 ml
10 mM inv-F	25 ml
10 mM inv-R	25 ml
10 mM pla-F	25 ml
10 mM pla-R	25 ml
10 mM yopM-F	25 ml
10 mM yopM-R	25 ml
H_2O	172.5 ml
TaKaRa Ex Taq (5 units/μl)	2.5 ml

The reaction mixture was devied into 10 tubes (24 ml / tube, 0.2 ml PCR tube). One ml of the template DNA prepared above section was added into each tube.

PCR condition

94 °C, 5 min
94 °C, 30 sec
55 °C, 30sec
72 °C, 60 sec
] 30 cycles

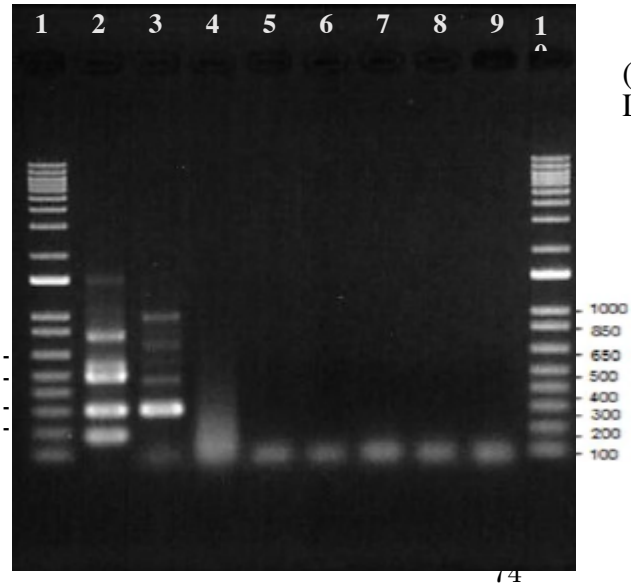
Electrophresis

An aliquot (5 ml) was applied to 1.5 % agarose gel. Ethidium bromide was used to visualize DNA in agarose gels.

Interpretation

Four amplified products: 171 bp (*caf1*), 295 bp (*inv*), 480 bp (*pla*) and 565 bp (*yopM*) can be observed in case of *Y. pestis* while only one band of 295 bp (*inv*) is found with *Y. pseudotuberculosis.* No band is amplified with the samples of other bacteria such as *Y. enterocolitica*.

< Typical result of multiplex PCR for identification of *Y. pestis*>



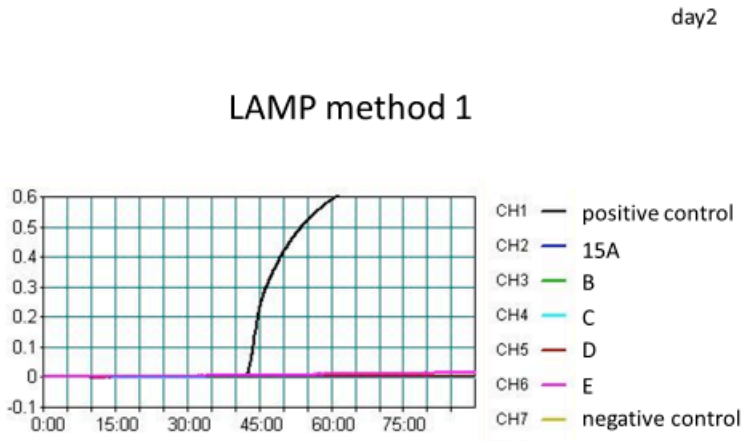
(Results)
Lanes 1, DNA markers
2, *Y. pestis* (+)
3, *Y. pseudotuberculosis* (-)
4, *Y. enterocolitica* (-)
5, Sample 15A (-)
6, Sample 15B (-)
7, Sample 15C (-)
8, Sample 15D (-)
9, Sample 15E (-)
10, DNA markers

資料 5

LAMP method 1

The LAMP method was performed according to the method described by Chantratita N. *et al.* (J Clin Microbiol. 2008; 46 (2): 568-73).

This method is briefly described below:
The LAMP reaction was performed using the Loopamp DNA amplification kit (Eiken Chemical Co., Ltd, Tokyo, Japan). The LAMP master mix contained 12.5 µl of 2X reaction mix, 5 pmol of each of the outer primers (F3, 5'-TCCTGCTCCCACACCG-3' and B3, 5'-GCTTGCGCGTCAT-3'), 40 pmol of each of the inner primers (FIP, 5'-CCACAGCAACGGAAAGAGCAGAGCTTGCGGCCGAGATC-3' and BIP, 5'-GCCTCGATTGCGCGATCGCCTGTTGCTAGCGGATTGTCA-3'), and 1 µl of *Bst* DNA polymerase.
The DNA samples were heated at 95°C for 5 min, before being ice-cooled and used as a template. *Burkholderia pseudomallei* DNA was used for the positive control, and distilled water was used for the negative control. Samples of 5 µl were used for each treatment.
The reagents and primers for each sample were mixed. The total reaction volume was 25 µl; this volume was maintained by reducing the volume of sterile distilled water added.
The amplification was performed at 65°C for 90 min, followed by incubation at 95°C for 2 min to terminate the reaction. The LAMP reaction causes turbidity in the reaction mix that is proportional to the amount of amplified DNA. The degree of turbidity was measured using an LA-320C Realtime Turbidimeter (Eiken), and the results were plotted on a graph.

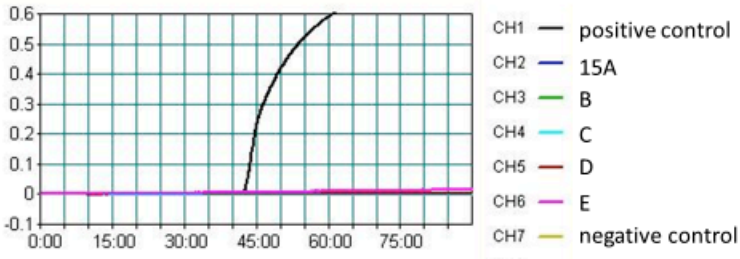


positive control; *B. pseudomallei* purified DNA
negative control; D.W.

J Clin Microbiol. 2008 Feb;46(2):568-73.
Chantratita N. et al.

day2

LAMP method 1



positive control; *B. pseudomallei* purified DNA
negative control; D.W.

J Clin Microbiol. 2008 Feb;46(2):568-73.
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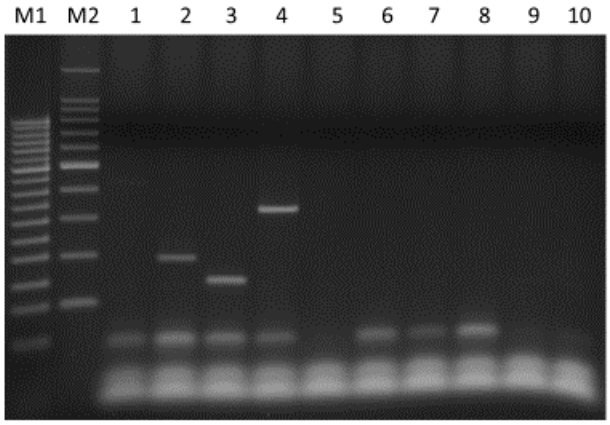
Multiplex PCR

Day2-day4



1: *Burkholderia mallei*
2: *Burkholderia pseudomallei*
3: *Burkholderia thailandensis*
4: *Burkholderia cepacia*
5:15A
M1: 50bp ladder
6: B
7: C
8: D
9: E
10: negative control (D.W.)
M2: 100bp ladder

Journal of Clinical Microbiology, 2011. P814-821. Vol49, No.3, Ho et al.

資料 6

PCR for detecting *Francisella tularensis*

Amplify and detect the gene sequences specific to *Francisella* spp. (16S rRNA) and the gene sequences specific to *Francisella tularensis*, *Francisella novicida*, and *Francisella hispaniensis* (*fop*A and *tul*4) by conventional PCR. If all 3 genes were amplified from the samples, PCRs for further discriminating subspecies *tularensis* or *holarctica*, with a pair of primers that amplifies the ISFtu2 sequence and the RD1 sequence are required.

Primer pairs

16S rRNA-PCR Forsman M. et al. (1994)

Forward primer F5 (5' to 3') CCTTTTTgAgTTTCgCTCC

Reverse primer F11 (5' to 3') TACCAGTTggAAACgACTgT

Approximate size of amplicon 1,100 bp

*fop*A-PCR Higgins J. A. et al. (2000)

Forward primer MS1 (5' to 3') CAgCTACTACACAAAgCAgTgg

Reverse primer MAI (5' to 3') CACCATTTACTgTATAgCACgC

Approximate size of amplicon 700 bp

*tul*4-PCR Sjöstedt A. et al. (1997)

Forward primer TUL4-435 (5' to 3') gCTgTATCATCATTTAATAAACTgCTg

Reverse primer TUL4-863 (5' to 3') TTgggAAgCTTgTATCATggCACT

Approximate size of amplicon 400 bp

Program

[94°C, 5 min.] ⇒

[(94°C, 3 sec.→ 58℃ , 30 sec.→ 72°C, 30 sec.) x 35 cycles] ⇒

[72°C, 7min.] ⇒

[4°C, ∞]

【Used regents】

DW

puRe Taq Ready-To-Go PCR Beads (GE Healthcare)

Control DNA: *F. tularensis* subsp. *holarctica* NVF1 strain(1ng/μl)

Prepare reaction mixture（on ice）

Contents	1 reaction	8 reactions
DW	20.5 μL	164 μL
10 μM Forward primer	1 μL	8 μL
10 μM Reverse primer	1 μL	8 μL
Total	22.5 μL	180 μL

Mix the above reagents in a 1.5 mL microtube and then, centrifuge.

Turn `ON` the thermal cycler (ABI GeneAmp PCR System 9700) and set the program.

Dispense 22.5 μL of the prepared reaction solution into the tubes of puRe Taq Ready-To-Go PCR Beads containing reagents

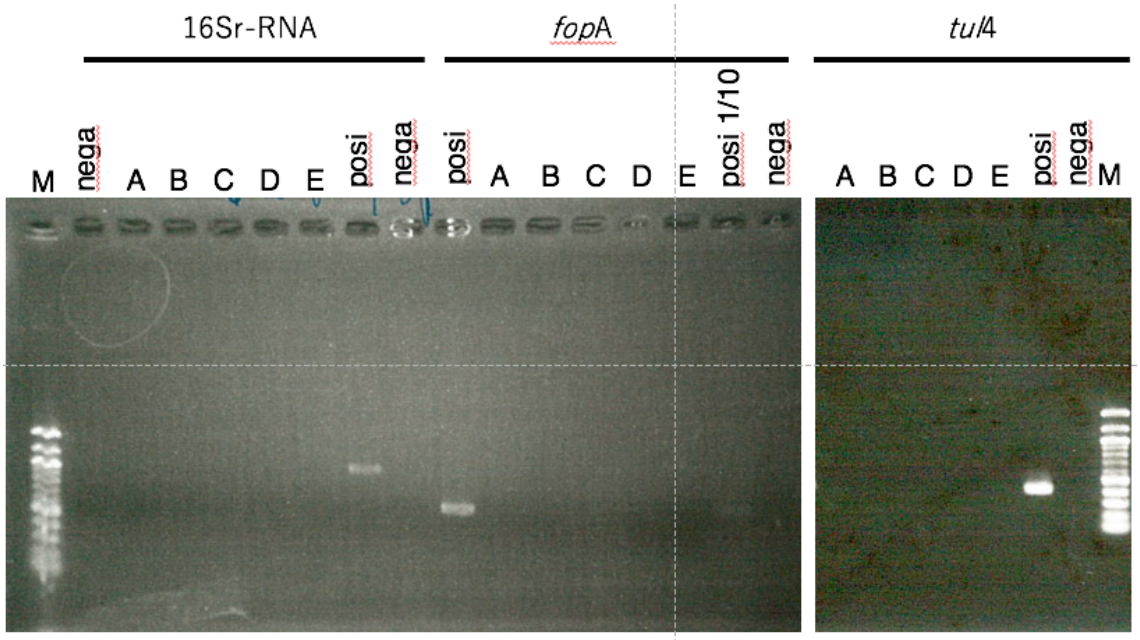
Add 2.5 μL each of the negative control, sample DNA, and positive control, and cap.

Set the PCR tube in the thermal cycler.

Start the program.

PCR products were electrophoresed in 1% agarose gel and were stained with ethidium bromide.

Results



M: 100 bp ladder marker
A, B, C, D, and E: Sample A, B, C, D, and E, respectively.
posi: *F. tularensis* DNA 1ng/ μl, posi 1/10: *F. tularensis* DNA 0.1ng/ μl
nega: D.W.

F. tularensis was not detected from all 5 samples, A, B, C, D, and E.

資料 7

Conventional PCR using WHO recommended primers for *Bacillus anthracis*

Day2

Primers

Target gene	Primer name	(5'-3')	Amplicon size
cap gene	CAP1234	CTGAGCCATTAATCGATATG	846 bp
	CAP1301	TCCCACTTACGTAATCTGAG	
pag gene	PA5	TCCTAACACTAACGAAGTCG	596 bp
	PA8	GAGGTAGAAGGATATACGGT	

PCR reaction solution

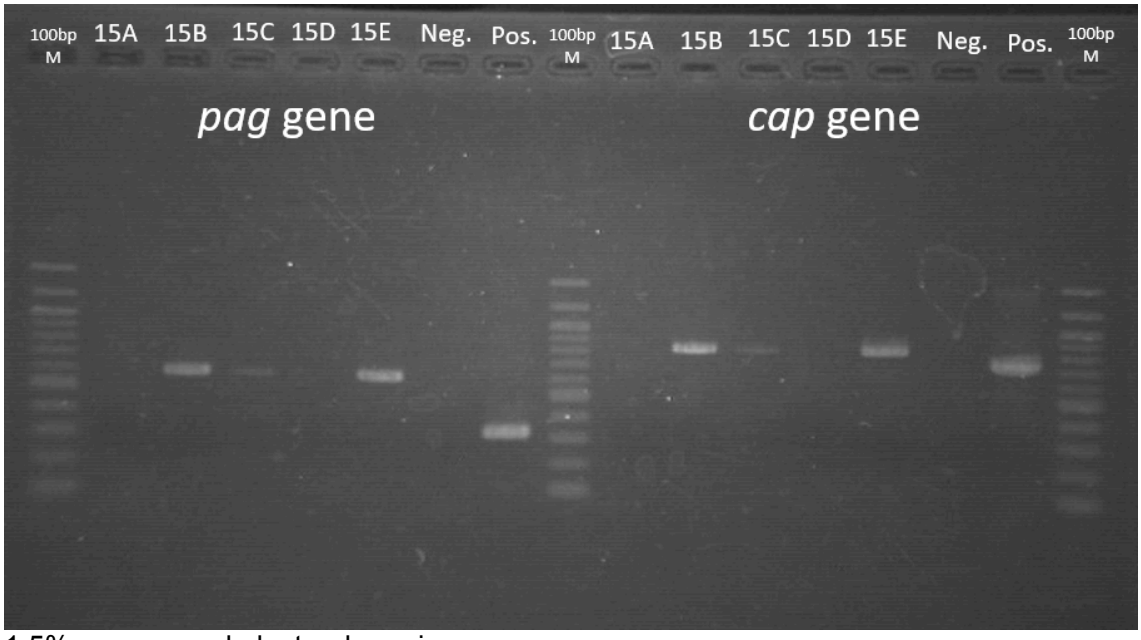
---TaKaRa ExTaq (TaKaRa, # RR001A)

Distrilled Water	33μl
10 x Buffer (TaKaRa Ex Taq)	5.0μl
dNTPs (2.5 mM each)	4.0μl
sense primer (10 pM)	1.0μl
anti-sense primer (10 pM)	1.0μl
TaKaRa ExTaq DNA Polymerase (2.5U/μl)	1.0μl
template	5.0μl
	total 50.0μl

PCR reaction (GeneAmp PCR System 9700)

(a) denaturing：95°C 5min
(b) 30 cycles
Denaturing: 95°C, 30 s
Annealing: 55°C, 30 s
Extension: 72°C, 30 s
(c) extension: 72°C, 5 min
4 min hold

Results



1.5% agarose gel electrophoresis
15A- 15E: Sample number
Neg.: Negative control (Distilled Water)

Pos.: Positive control for PCR (shortened size inserted plasmid DNA)
15B, 15C and 15E had positive PCR amplification.
pag gene 596 bp, positive control 322 bp. *cap* gene 846 bp, positive control 720 bp.

Day3-4
BLASTN of sequence of PCR amplicons.
100 % identity to *B. anthracis* pag gene
15B-pag, 15C-pag, 15E-pag amplicon
100 % identity to *B. anthracis cap* gene
15B-cap, 15C-cap, 15E-cap amplicon

Sample number	PCR amplification		Identified as *B. anthracis*
	cap	*pag*	
15A	–	–	negative
15B	+	+	positive
15C	+	+	positive
15D	–	–	negative
15E	+	+	positive

16S rRNA amplification
Day2
PCR for sequencing template preparation
16S rRNA primers

1. 27f (8-27) -r2L (821-803) 814bp
2. f2L (518-536) -1492r (1510-1492) 993bp

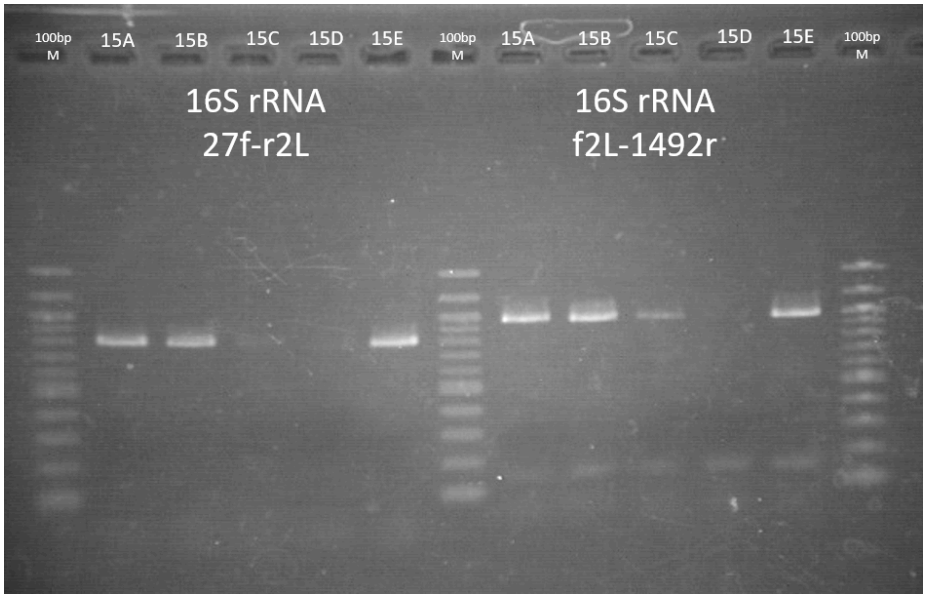
PCR sample preparation

DEPC-H_2O	17.5μL
NEBNext High-Fidelity 2X PCR Master Mix (NEB, #M0541S)	25uL (0.02U/μL, 0.2mM)
Primer Forward (10μM)	2.5μL (0.5μM)
Primer Reverse (10μM)	2.5μ L (0.5μM)
Sample DNA (1 ng/μL≤)	2.5μL
	Total volume 50μL

PCR reaction (GeneAmp PCR System 9700)

(a) denaturing: 98 °C 30sec
(b) 30 cycles
denaturing: 98 °C, 10s
annealing: 62 °C, 30s
extension: 72 °C, 30s
(c) extension: 72 °C, 5min
4 min hold

Results



1.5% agarose gel electrophoresis
15A- 15E: Sample number
15A, 15B, 15C and 15E had positive PCR amplification (both genes) of expected sizes.

Day3-4
BLASTN of sequence of PCR amplicons.

15A-r2L
100 % identity to *Bacillus cereus, B. thruringiensis, B. toyonensis* 16S rRNA gene (36-725bp)
15B-r2L
100% identity to *B. anthracis, B. cereus* 16S rRNA gene (11-749bp)
15C
could not identify because of low qualities of sequences
15E-r2L
100% identity to *B. anthracis, B. cereus* 16S rRNA gene (24-750bp)

Sample number	PCR amplification		BLASTN results
	27f-r2L	f2L-1492r	
15A	+	+	*B. cereus, B. thruringiensis, B. toyonensis*
15B	+	+	*B. anthracis, B. cereus*
15C	+	+	could not be identified
15D	–	–	–
15E	+	+	*B. anthracis, B. cereus*