Activity report from Hokkaido University
- Progress of R/D and tech transfer on glanders -

Faculty of Veterinary Medicine

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JCC Nov 17' 2022

Glanders

R/D items, methods and milestones

1. Development of rapid diagnostic method for *B. mallei* infection by LAMP method

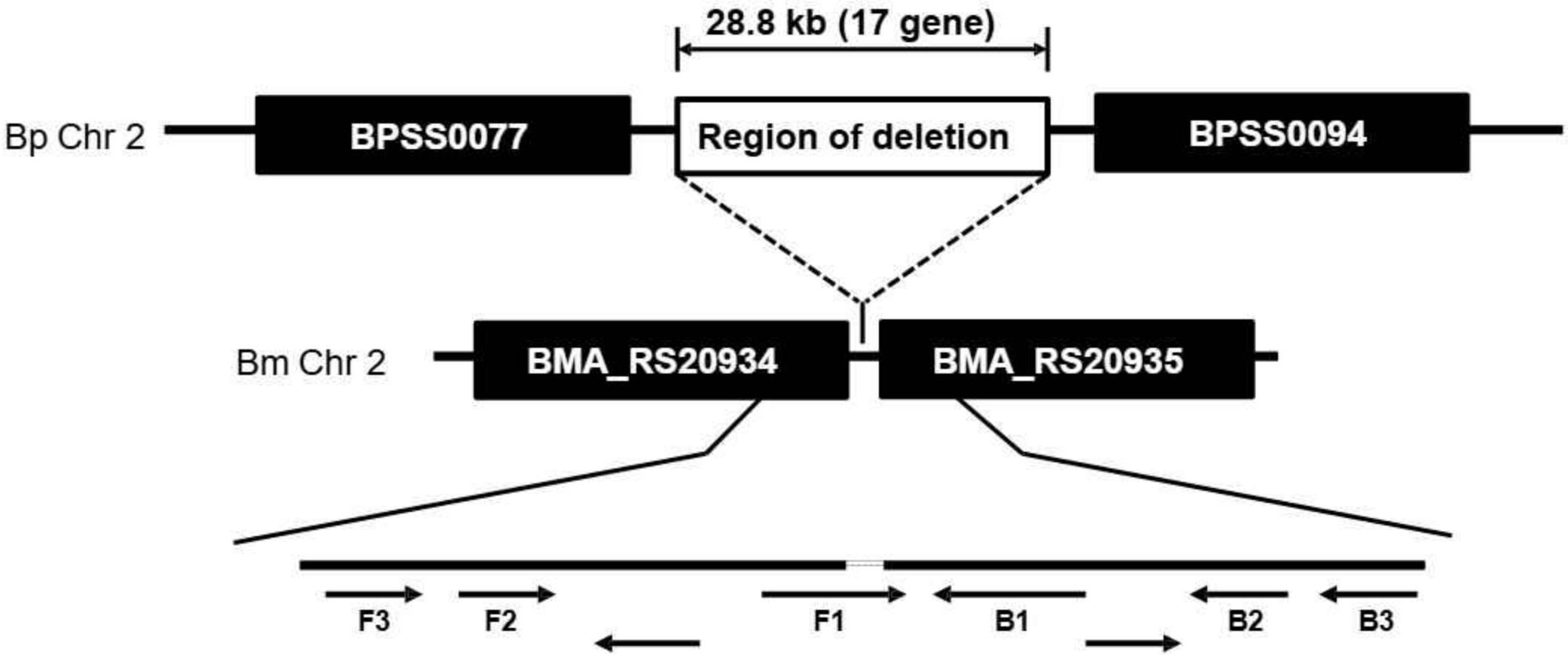
2. Development of rapid diagnostic method for *B. mallei* infection by immunochromatography

3. Understanding the prevalence of animal glanders and strengthening the foundation for epidemic prevention

1. Development of rapid diagnostic method for *B. mallei* infection by a loop-mediated isothermal amplification (LAMP)

Dai Hasegawa, Thoko Flav Kapalamula, Yasuhiko Suzuki, Takashi Kimura, Chie Nakajima

Target region



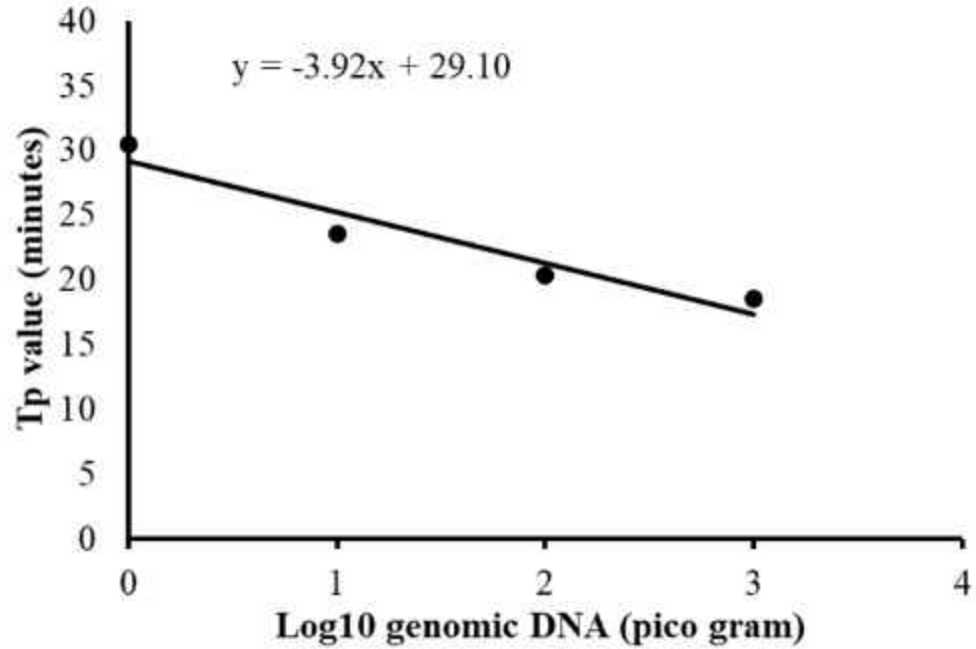
Bm genome 1.0 pg = 159 copies

Sensitivity of *B. mallei*-specific LAMP method

(A)

Amount of DNA (pg) per reaction	1,000	100	10	1	0.5	0.25
Tp value (min:sec)	18:48	20:36	23:54	30:48	40:12	—

(B)



Correlation between template amount of serially diluted *B. mallei* GTC 3P0003T genomic DNA and positive detection time (Tp value: Time to positivity). ー：negative。

Standard curve drawn by plotting the Tp value against the logarithmic value of the amount of genomic DNA.

Development of rapid diagnostic method for *B. mallei* infection by LAMP

Research progress

- Targeting the 28.8 kbp consensus sequence that is missing in *B. mallei* compared to *B. pseudomallei*, we developed *B. mallei* LAMP, which enables discrimination between *B. mallei* and *B. pseudomallei*.

Plans for this year

- Introduce an ink jet printer to NCCD for LAMP production.

- Establish a method that can detect *B. mallei* with higher detection sensitivity.

- Prepare dried-form of *B. mallei* LAMP using an inkjet printer installed at the Hokkaido University International Institute for Zoonosis Contorl.

Development of rapid diagnostic method for *B. mallei* infection by LAMP

Research progress

- Targeting the 28.8 kbp consensus sequence that is missing in *B. mallei* compared to *B. pseudomallei*, we developed *B. mallei* LAMP, which enables discrimination between *B. mallei* and *B. pseudomallei*.

Plans for this year

- Introduce an ink jet printer to NCCD for LAMP production.

→ The printer itself has already been sent to NCCD by air. The clean booth sent by rail is scheduled to arrive by the end of March, 2023.

- Establish a method that can detect *B. mallei* with higher detection sensitivity.

→ Conditions for the above mentioned *B. mallei*-specific LAMP have been completed. In addition, we are currently developing a more sensitive LAMP method to detect *B. mallei* and *B. pseudomallei*, which cannot be distinguished from each other.

- Prepare dried-form of *B. mallei* LAMP using an inkjet printer installed at the Hokkaido University International Institute for Zoonosis Contorl.

2. Development of rapid diagnostic method for *B. mallei* infection by immunochromatography

Yoshiki Ichikawa, Tomohiro Okagawa, Yukiho Iinuma, Liushiqi Borjigin,
Kazukiko Ohashi, Takashi Kimura

Search for new candidate antigens for immunochromatographic test (ICT)

- Antigens were selected with reference to studies that used *B. pseudomallei* protein microarrays to screen for the reactivity with human patient sera.

Analysis of serodiagnostic antigens based on Bp protein microarray

Report	Year	Sera screened	Patient's country
Felgner PL, et al. PNAS	2009	Melioidosis (humans)	Singapore, Thailand, USA
Suwannasaen D, et al. J Infect Dis	2011	Melioidosis (humans)	Thailand
Varga JJ, et al. Virulence	2012	Glanders (human)	USA
Kohler C, et al. PLoS Negl Trop Dis	2016	Melioidosis (humans)	Thailand

21 Bm antigens with serodiagnostic potential + Whole bacterial lysate

	Bm locus ID	Protein	kDa	Signal	TMHM
Glanders specific antigens in the report of 2012	BMA1071	hypothetical protein	7	-	-
	BMAA1630	type III, inner membrane SctV	74	-	308-690
	BMA2431	chaperonin, 10 kDa	11	-	-
	BMAA0633	putative outer membrane porin	39	23-379	-
	BMA2073	hypothetical protein	19	-	36-186
Antigens with high AUC in the report of 2009	BMA2001	GroEL	57	-	-
	BMA0436	OmpA precursor	24	23-224	-
	BMA1487	antioxidant, AhpC/Tsa family	20	-	-
	BMAA0351	oligopeptide ABC transporter	61	-	44-554
	BMAA1523	type III, effector BopE	28	-	-
	BMA0434	putative exported protein	21	22-198	-
	BMA2002	chaperonin, 10 kDa	10	-	-
	BMAA0356	outer membrane porin	51	29-480	27-480
	BMAA1530	type III, target BipC	44	-	-
	BMAA1751	malate dehydrogenase	35	-	-
	BMAA0749	autotransporter, BimA	38	-	-
	BMAA1609	type 4 pilus biosynthesis protein	47	-	-
	BMA2642	50S ribosomal protein L7/L12	12	-	-
Commercially available ELISAs	BMAA0744	TssA	18	-	-
	BMAA0743	TssB	56	-	-
	BMAA0742	Hcp1	19	-	-

We were able to express 16 *B. mallei* antigens

	Bm locus ID	Protein	kDa	Fraction
Glanders specific antigens in the report of 2012	BMA1071	hypothetical protein	7	-
	BMAA1630	type III, inner membrane SctV	74	-
	BMA2431	**chaperonin, 10 kDa**	**11**	**Soluble**
	BMAA0633	putative outer membrane porin	39	(Inclusion)
	BMA2073	hypothetical protein	19	-
High AUC in the report of 2009	**BMA2001**	**GroEL**	**57**	**Soluble**
	BMA0436	**OmpA precursor**	**24**	**Soluble**
	BMA1487	**antioxidant, AhpC/Tsa family**	**20**	**Soluble**
	BMAA0351	**oligopeptide ABC transporter**	**61**	**Soluble**
	BMAA1523	**type III, effector BopE**	**28**	**Soluble**
	BMA0434	**putative exported protein**	**21**	**Soluble**
	BMA2002	**chaperonin, 10 kDa**	**10**	**Soluble**
	BMAA0356	outer membrane porin	51	-
	BMAA1530	**type III, target BipC**	**44**	**Soluble**
	BMAA1751	**malate dehydrogenase**	**35**	**Soluble**
	BMAA0749	**autotransporter, BimA**	**38**	**Inclus/Inclus**
	BMAA1609	**type 4 pilus biosynthesis protein**	**47**	**Inclusion**
	BMA2642	**50S ribosomal protein L7/L12**	**12**	**Soluble**
Commercially available ELISAs	**BMAA0744**	**TssA**	**18**	**Soluble**
	BMAA0743	**TssB**	**56**	**Soluble**
	BMAA0742	**Hcp1**	**19**	**Soluble**

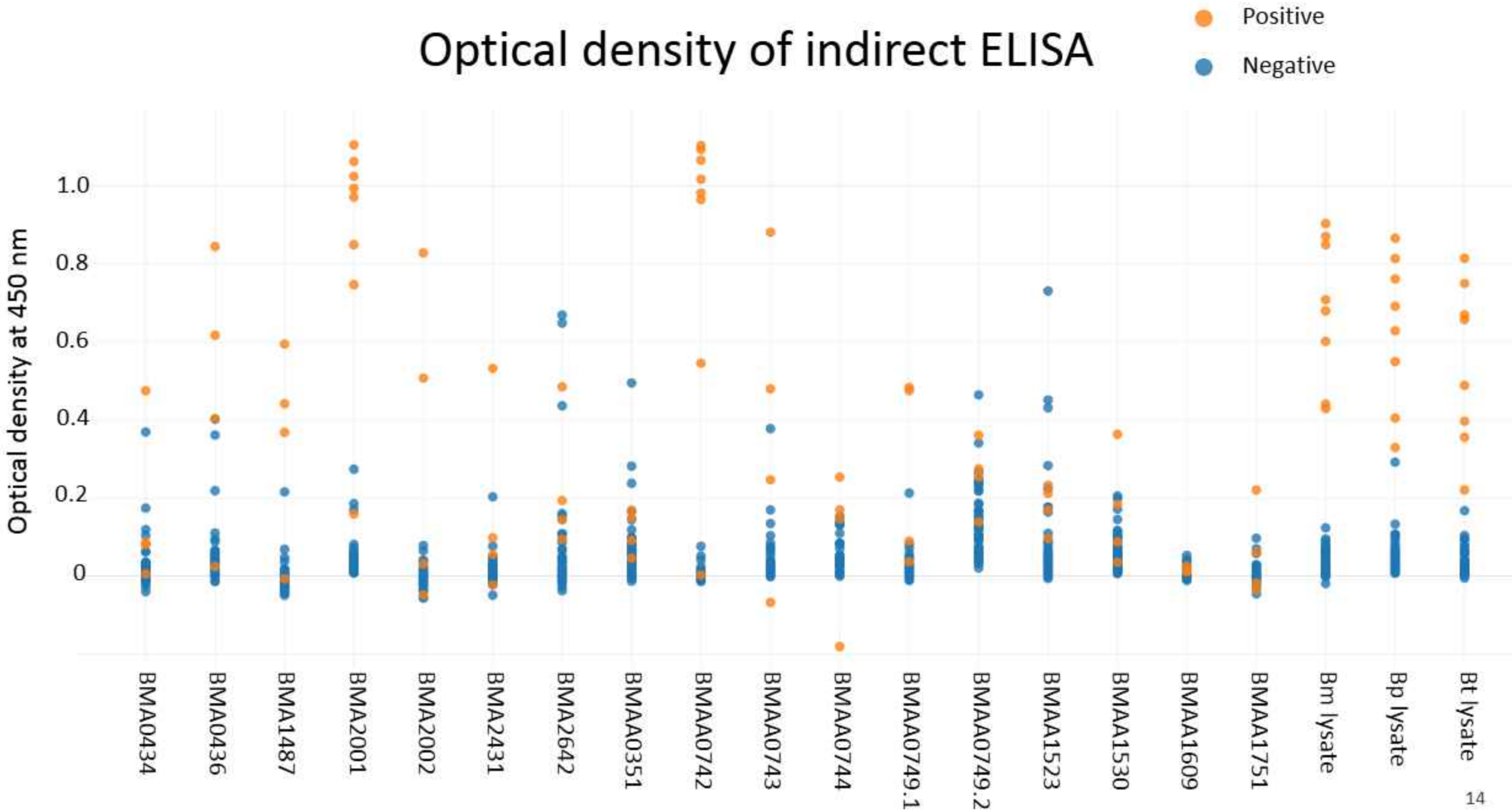
Indirect ELISA

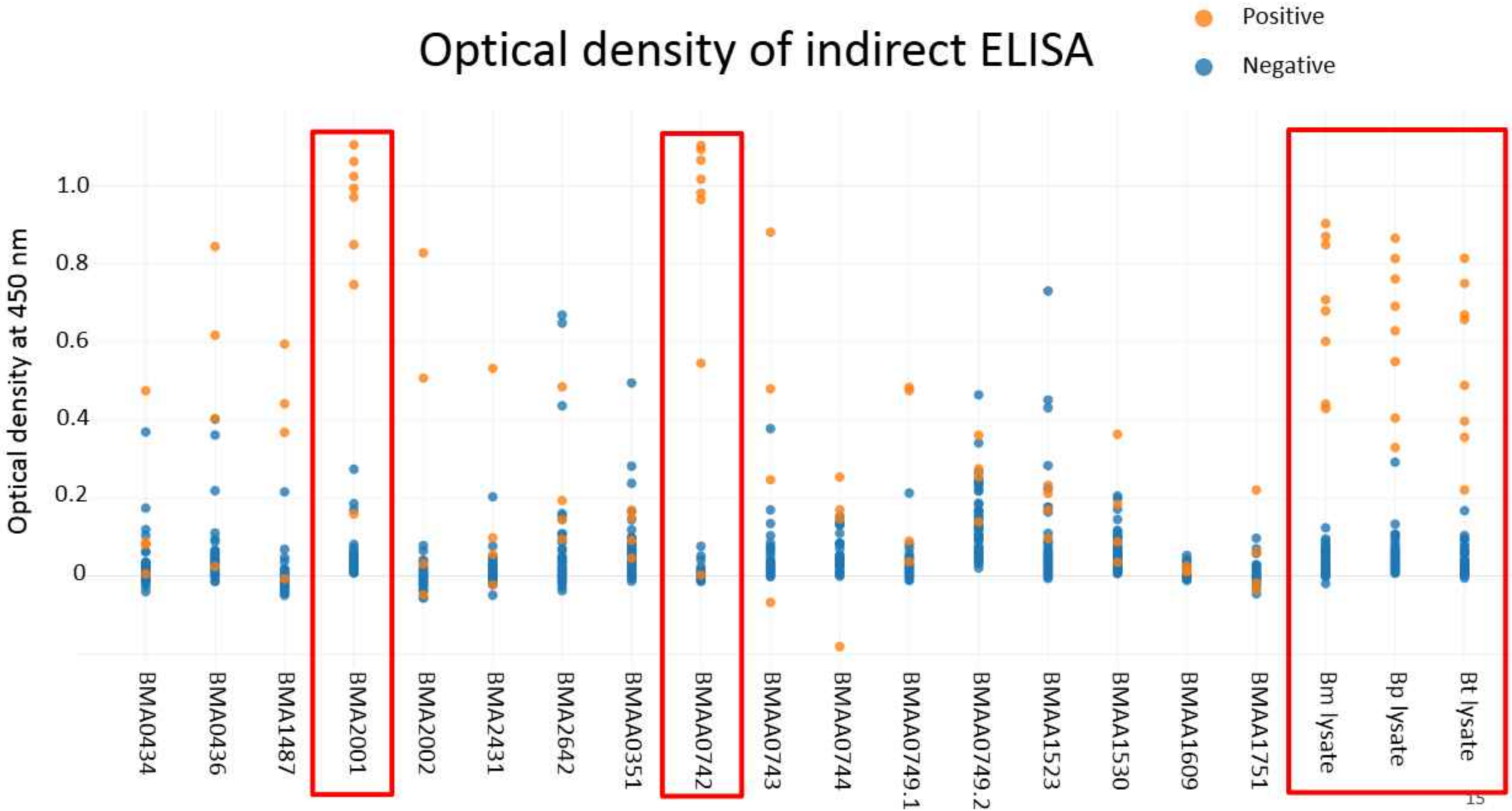
- Antigen: 6 × His tagged recombinant Bm proteins, whole bacterial lysate
- Antigen concentration: 1, 5, or 10 µg/ml in carbonate-bicarbonate buffer (pH 9.6)
- Blocking: 5% Skim milk in PBST
- 2^{nd} antibody: HRP conjugated anti-equine IgG antibody
- Substrate: TMB One Component, RT, 6 min

Horse sera

Glanders positive and negative sera were selected from the horse sera collected in Mongolia in 2018 and 2019

Rose Bengal test	CFT	Mallein test	
Positive	Positive	Positive	→ True positive serum samples
Negative	Negative	—	→ True negative serum samples


Optical density of indirect ELISA
Positive
Negative
Optical density at 450 nm
1.0
0.8
0.6
0.4
0.2
0
BMA0434
BMA0436
BMA1487
BMA2001
BMA2002
BMA2431
BMA2642
BMAA0351
BMAA0742
BMAA0743
BMAA0744
BMAA0749.1
BMAA0749.2
BMAA1523
BMAA1530
BMAA1609
BMAA1751
Bm lysate
Bp lysate
Bt lysate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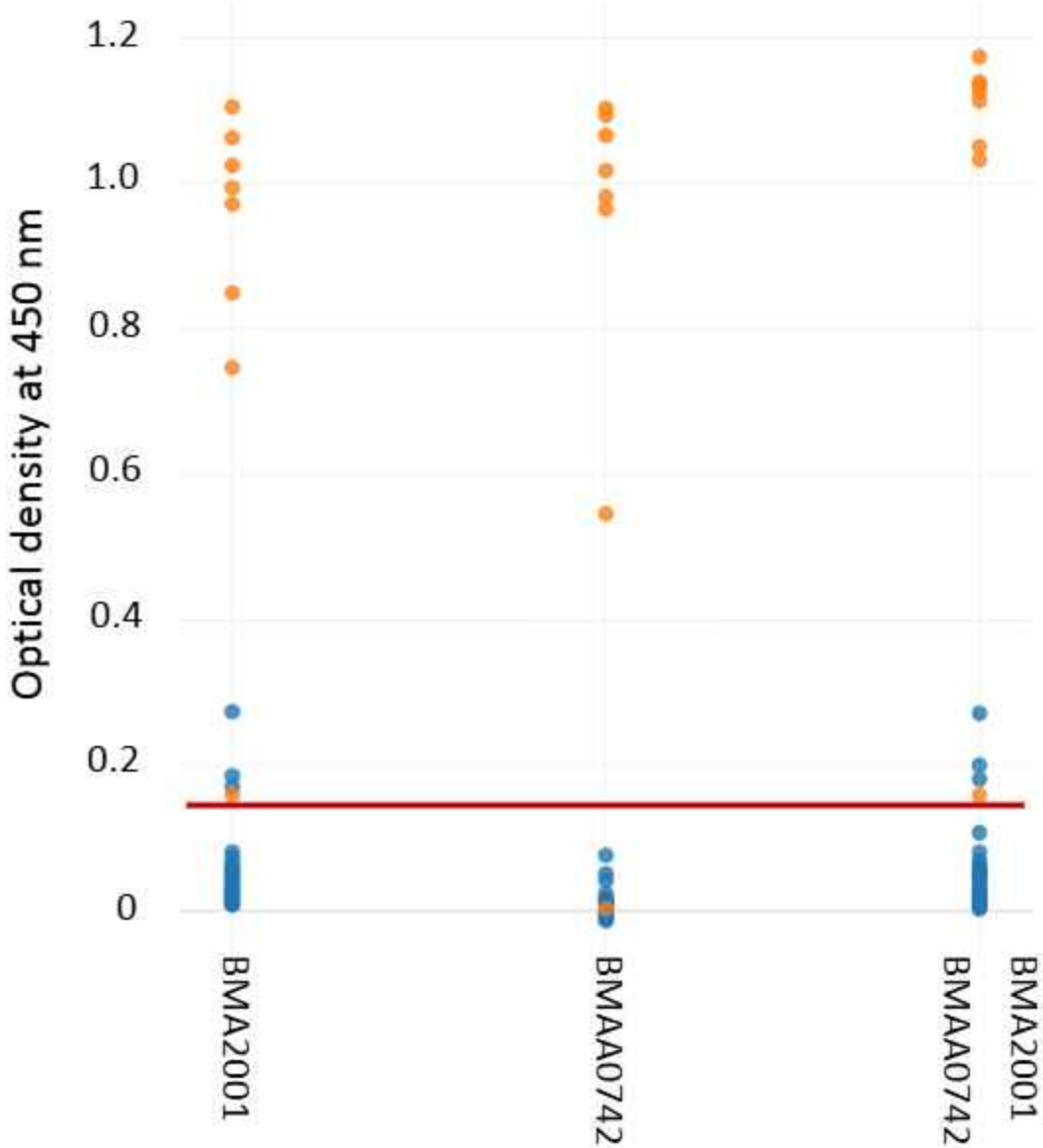
BMAA0742
BMAA0743
BMAA0744
BMAA0749.1
BMAA0749.2
BMAA1523
BMAA1530
BMAA1609
BMAA1751
Bm lysate
Bp lysate
Bt lysate

Indirect ELISA using mixed antigens



BMA2001 (GroEL)

Sensitivity: 100%

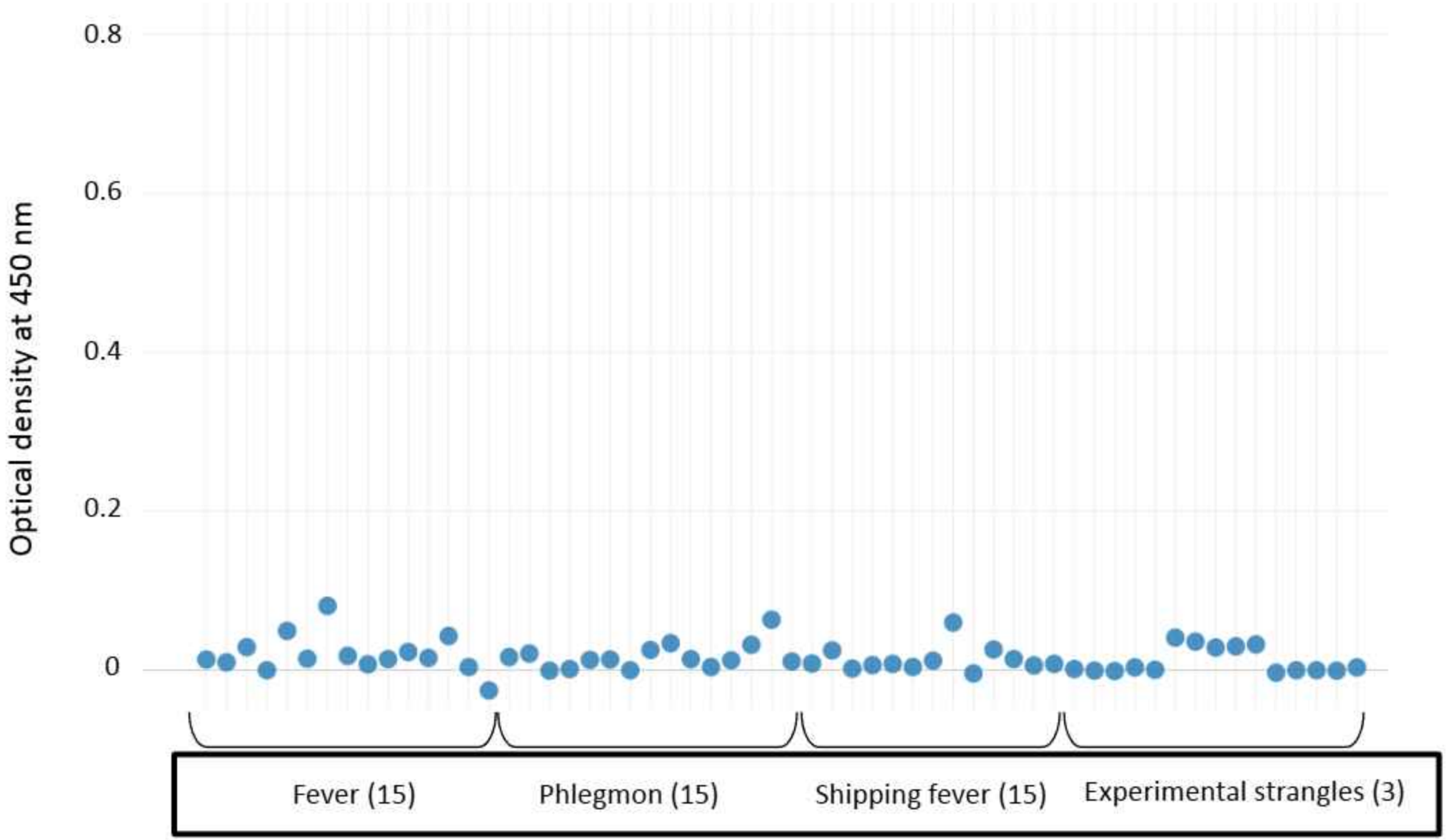
Specificity: 91.7%

BMAA0742 (Hcp1)

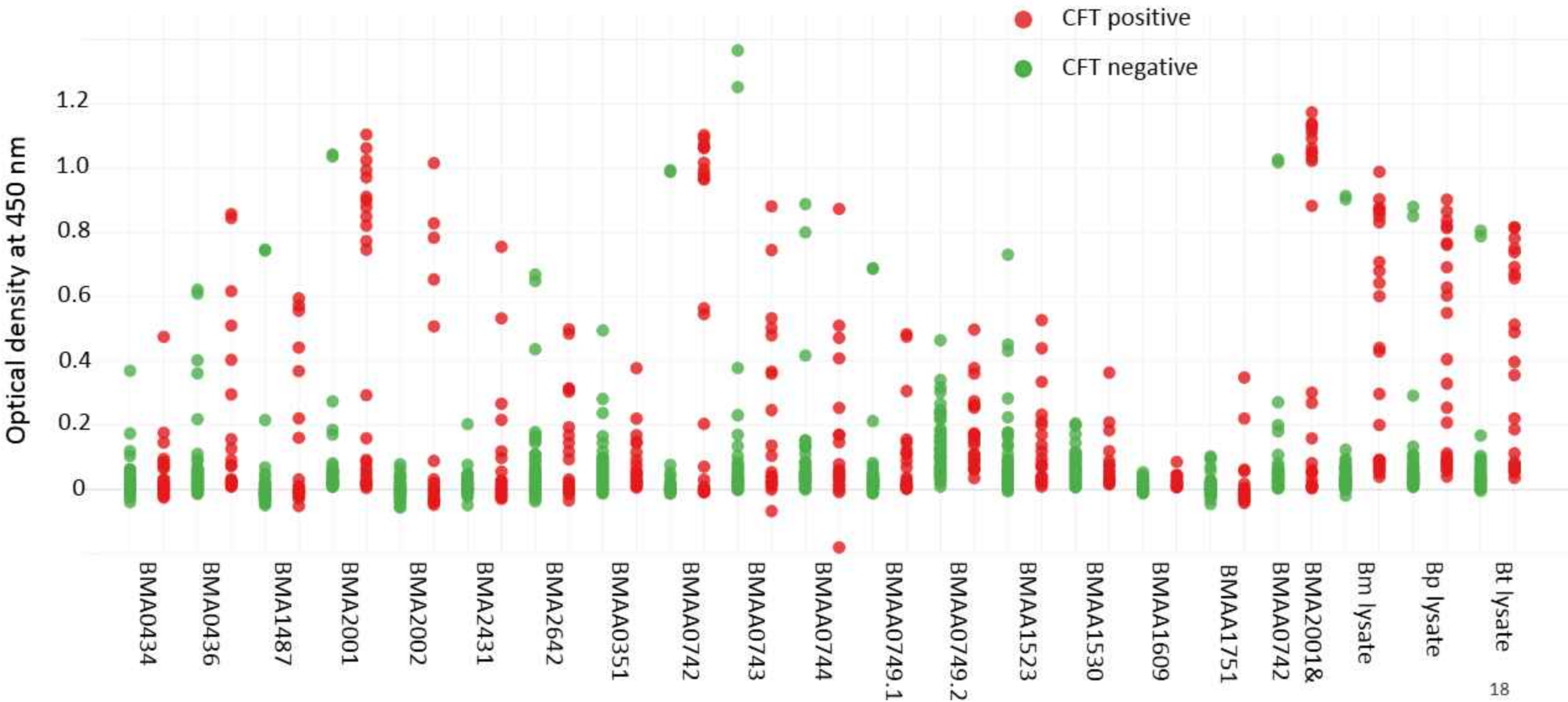
Sensitivity: 87.5%

Specificity: 100%

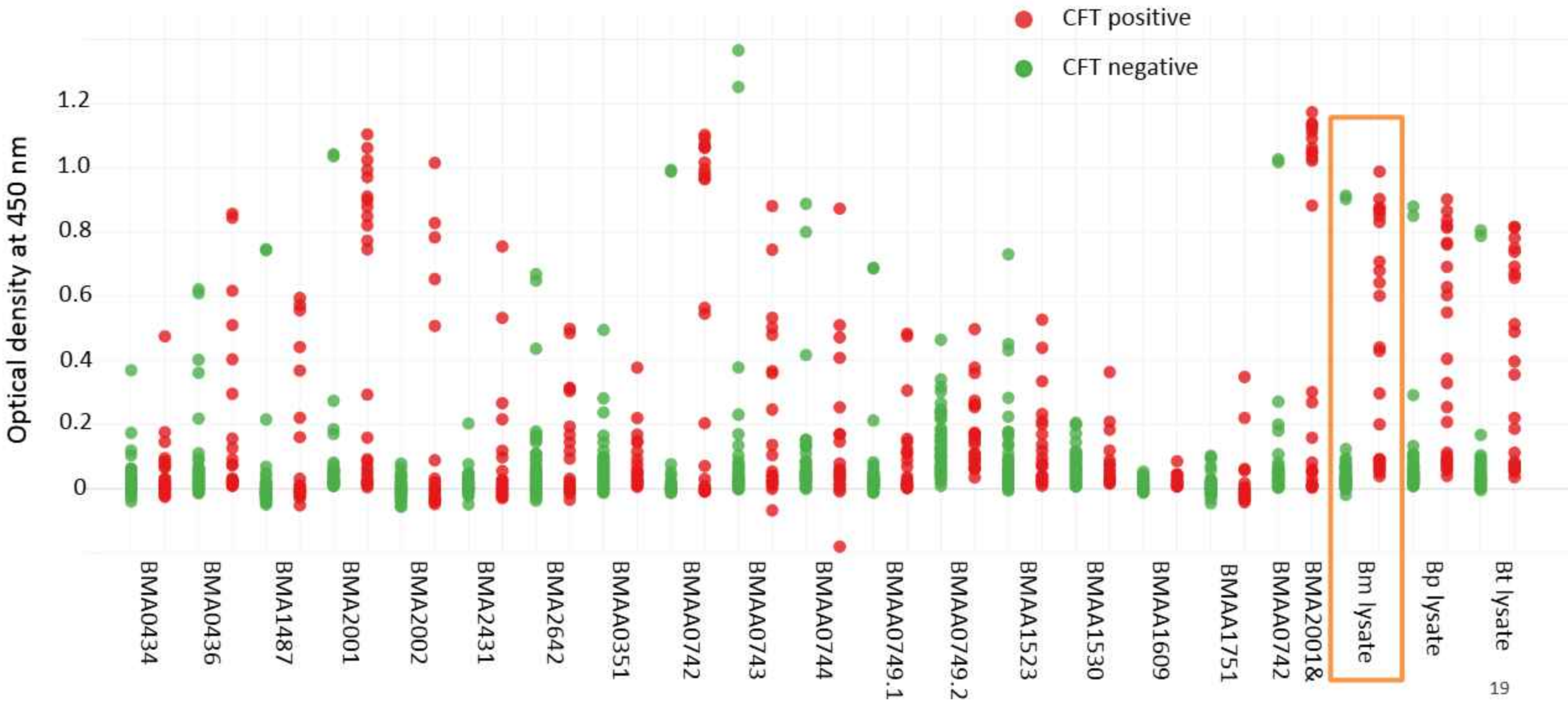
Whole Bm lysate do not react with negative serum collected in Japan



Comparison of indirect ELISA and CFT using 104 horse serum samples



Comparison of indirect ELISA and CFT using 104 horse serum samples



Comparison of indirect ELISA and CFT using 104 horse serum samples

ELISA using Bm lysate as antigen (cutoff = 0.25)

CFT		ELISA: Negative	ELISA: Positive
	Negative	76	2
	Positive	13	14

Comparison of indirect ELISA and CFT using 104 horse serum samples

ELISA using Bm lysate as antigen
(cutoff = 0.25)

CFT		Negative	Positive
	Negative	76	2
	Positive	13	14

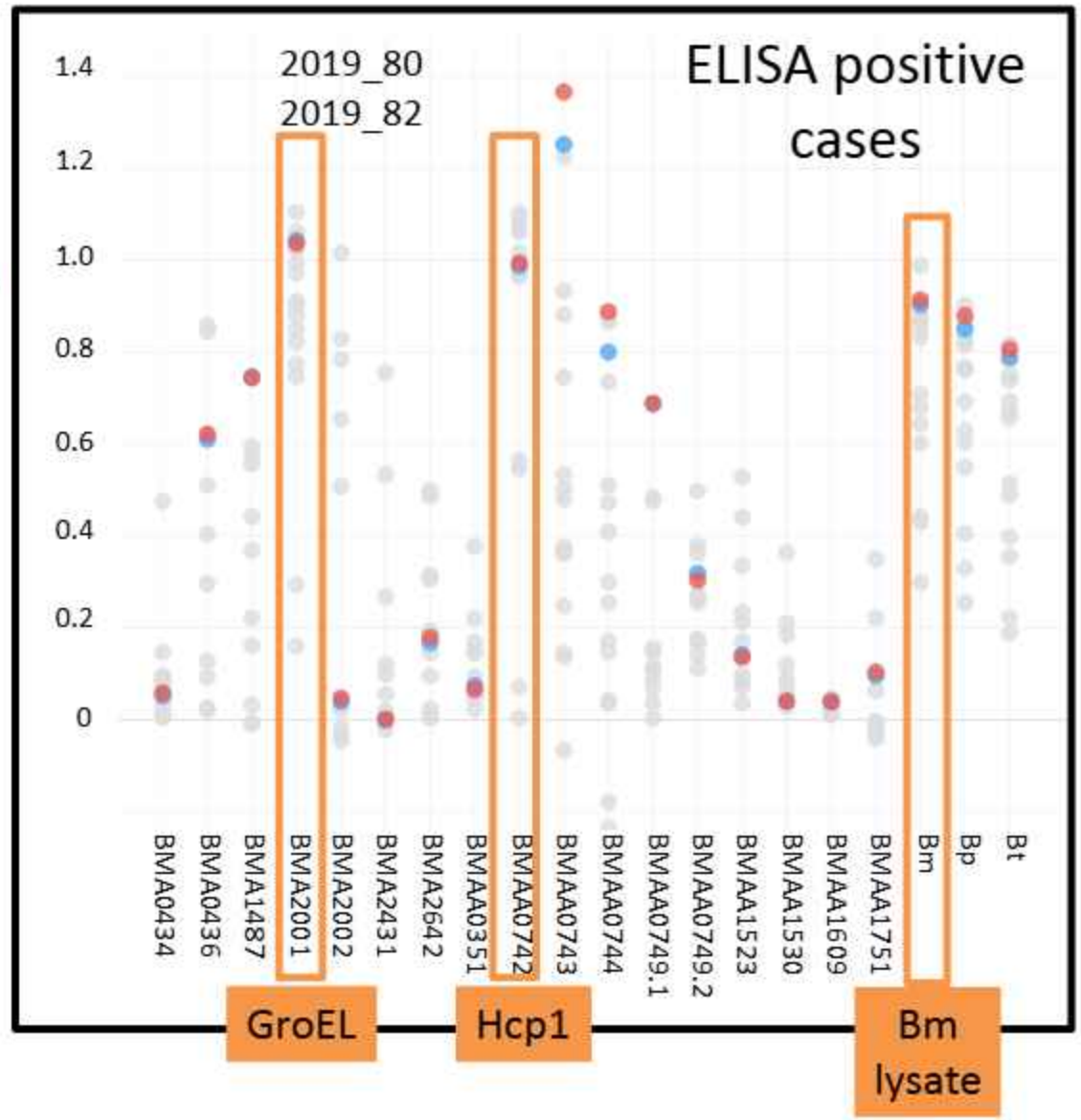
Year	Serum	RBT	CFT	Mallein	Clinical_sign	Bm
2019	1	P	P	P	Clinically	0.708
2019	2	P	P	N	-	0.831
2019	3	P	P	P	-	0.849
2019	4	P	P	N	-	0.2975
2019	5	P	P	N	-	0.877
2019	6	P	P	N	-	0.6425
2019	7	P	P	N	-	0.9885
2019	8	P	P	P	Clinically	0.903
2019	9	P	P	N	-	0.8635
2019	10	P	P	P	Clinically	0.441
2018	186	P	P	P	Clinically	0.6795
2018	187	P	P	P	Clinically	0.4295
2018	188	P	P	P	Clinically	0.6015
2018	189	P	P	P	Clinically	0.87

Same herd

Comparison of indirect ELISA and CFT using 104 horse serum samples

ELISA using Bm lysate as antigen
(cutoff = 0.25)

CFT		Negative	Positive
	Negative	76	2
	Positive	13	14



Comparison of indirect ELISA and CFT using 104 horse serum samples

ELISA using Bm lysate as antigen
(cutoff = 0.25)

CFT		Negative	Positive
	Negative	76	2
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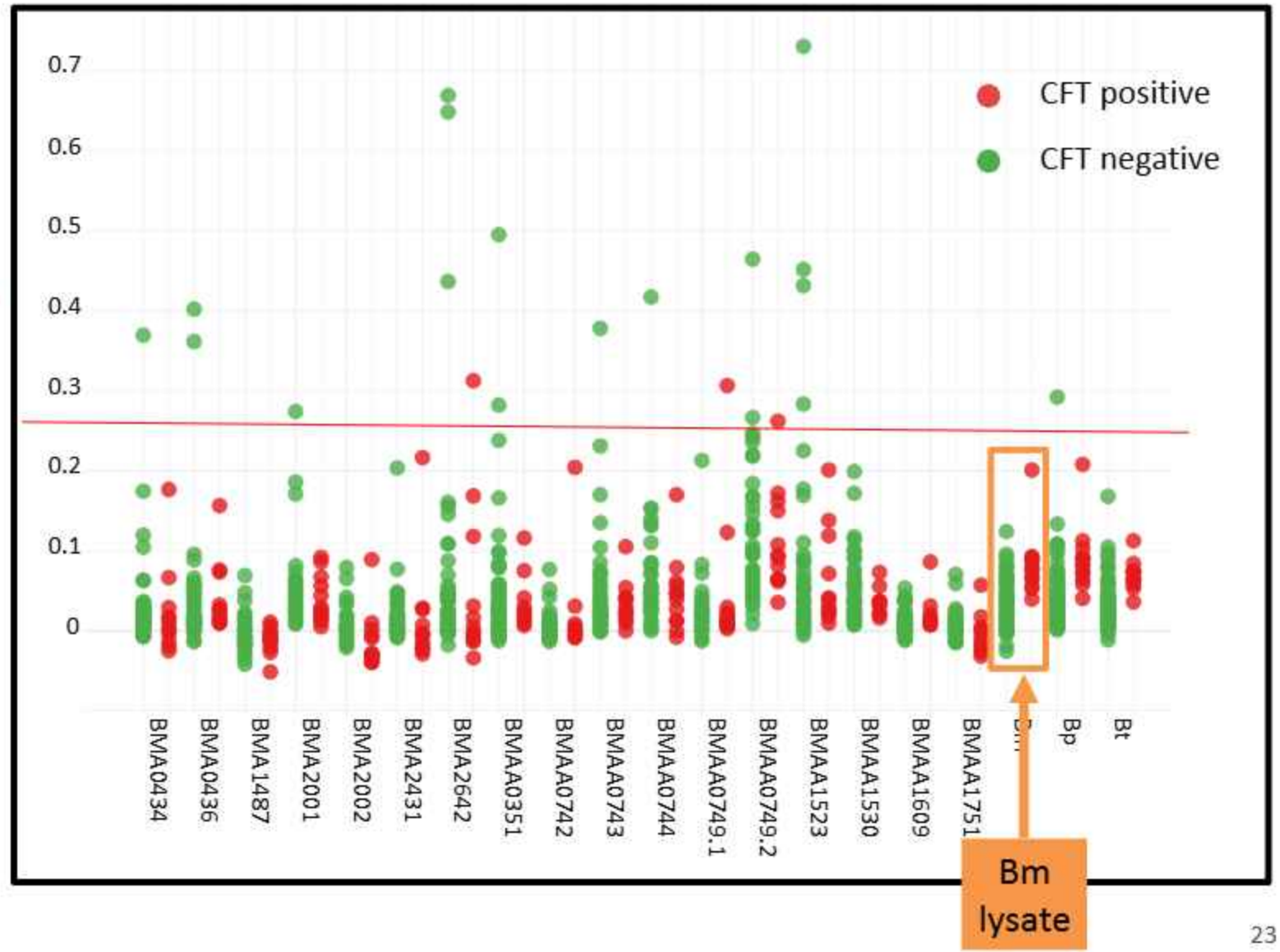


Development of rapid diagnostic method for *B. mallei* infection by immunochromatography

Research progress

- By examining the reactivity of 16 recombinant *B. mallei* proteins with glandrous horse serum collected in Mongolia, we have narrow down the candidate antigens to be used for immunochromatographic test (ICT) to three (GroEL, Hcp1, *B. mallei* lysate).

Plans for this year

- Test horse sera (>1,000) collected in 2021 and 2022 from Tuv, Uvurkhangai, Dundgobi, Sukhbaatar, Khobd, Selenge, Dornod, Khentii, Umnugobi provinces by an indirect ELISA using the three selected antigens.

- Make prototype immunochromatography using three antigens

3. Understanding the prevalence of animal glanders and strengthening the foundation for epidemic prevention

Liushiqi Borjigin, Yoshiki Ichikawa, Kazuhiko Ohashi, Yasuhiko Suzuki, Takashi Kimura

Research progress

- Dr. Liushiqi stayed in Mongolia from October 2021 to February 2022 and introduced methods for isolating *Mycobacterium bovis* and *B. mallei* from animal tissue.

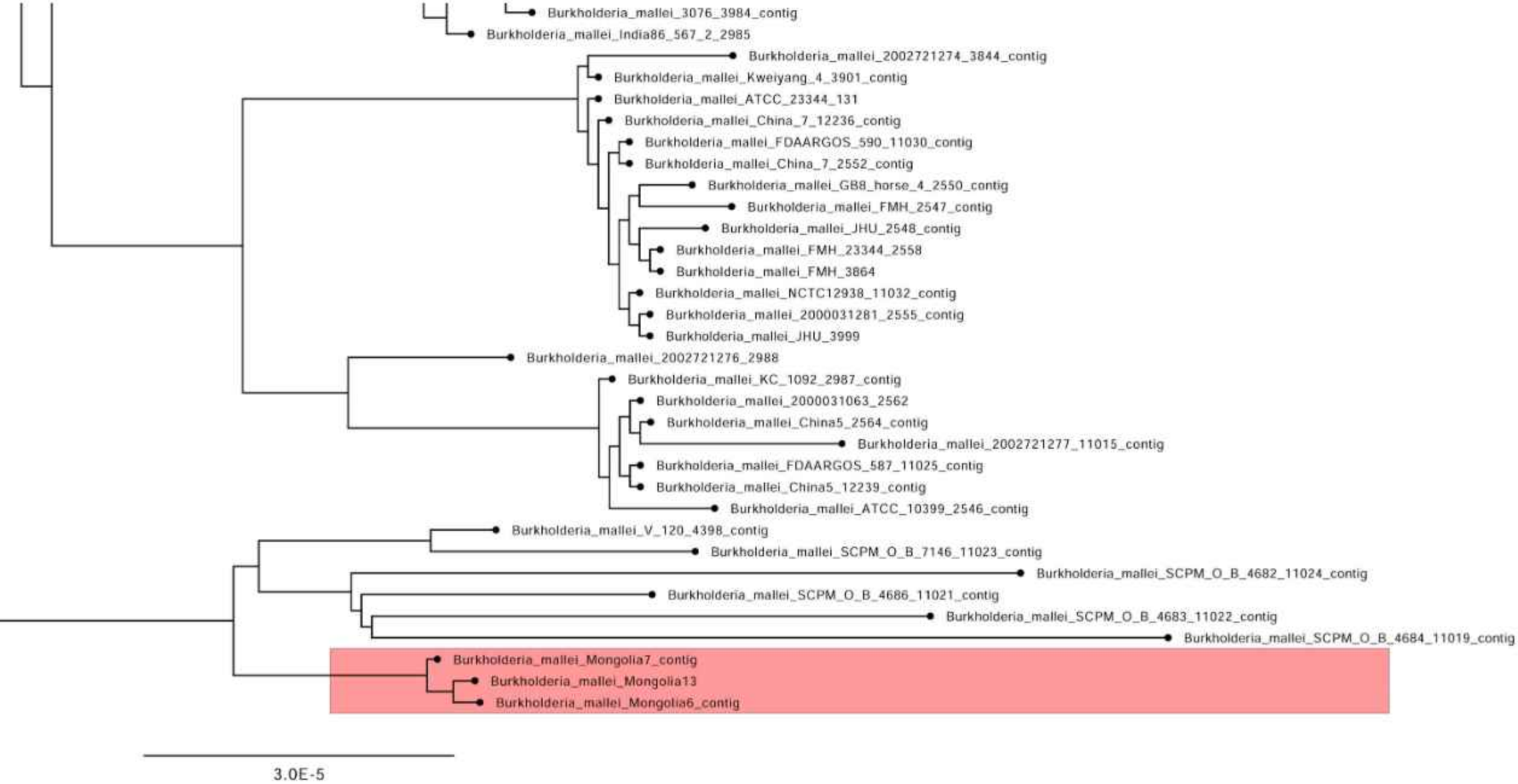
- Construction of an interferon gamma release assay (IGRA) for glanders was initiated by Dr. Liushiqi.

- Dr. Liushiqi and Dr. Ichikawa stayed in Mongolia from April 22 to July 22 or from June 10 to July 4, respectively, and participated in an epidemiological survey in Dundgobi, Uvurkhangai and Selenge.

- Dr. Kimura and Dr. Ichikawa stayed in Mongolia from September 16 to October 3 and participated in an epidemiological survey in Selenge.

- Three strains of *B. mallei* isolated by IVM were cultured from single colonies on selective media, and the extracted DNA was analyzed by whole-genome sequencing at Hokkaido University.

- From November 2022 to January 2023, a total of 6 young researchers from IVM will be accepted for training on the diagnosis of animal tuberculosis and glanders at Hokkaido University.


Burkholderia_mallei_3076_3984_contig
Burkholderia_mallei_India86_567_2_2985
Burkholderia_mallei_2002721274_3844_contig
Burkholderia_mallei_Kweiyang_4_3901_contig
Burkholderia_mallei_ATCC_23344_131
Burkholderia_mallei_China_7_12236_contig
Burkholderia_mallei_FDAARGOS_590_11030_contig
Burkholderia_mallei_China_7_2552_contig
Burkholderia_mallei_GB8_horse_4_2550_contig
Burkholderia_mallei_FMH_2547_contig
Burkholderia_mallei_JHU_2548_contig
Burkholderia_mallei_FMH_23344_2558
Burkholderia_mallei_FMH_3864
Burkholderia_mallei_NCTC12938_11032_contig
Burkholderia_mallei_2000031281_2555_contig
Burkholderia_mallei_JHU_3999
Burkholderia_mallei_2002721276_2988
Burkholderia_mallei_KC_1092_2987_contig
Burkholderia_mallei_2000031063_2562
Burkholderia_mallei_China5_2564_contig
Burkholderia_mallei_2002721277_11015_contig
Burkholderia_mallei_FDAARGOS_587_11025_contig
Burkholderia_mallei_China5_12239_contig
Burkholderia_mallei_ATCC_10399_2546_contig
Burkholderia_mallei_V_120_4398_contig
Burkholderia_mallei_SCPM_O_B_7146_11023_contig
Burkholderia_mallei_SCPM_O_B_4682_11024_contig
Burkholderia_mallei_SCPM_O_B_4686_11021_contig
Burkholderia_mallei_SCPM_O_B_4683_11022_contig
Burkholderia_mallei_SCPM_O_B_4684_11019_contig
Burkholderia_mallei_Mongolia7_contig
Burkholderia_mallei_Mongolia13
Burkholderia_mallei_Mongolia6_contig
3.0E-5

Future plan in FY2023

- Create a prototype immunochromatography and examine its usefulness using sera collected in epidemiological survey.
- Develop IGRA for glanders and examine its usefulness in epidemiological survey.
- Examine the usefulness of LAMP using nasal discharge/skin nodular exudate collected in epidemiological survey.



Progress on JICA ODA activities

- On March 23, 2022, Mr. Toshiro Sato, Coordinator, arrived in Mongolia.
- Of the equipment (R/D Annex 8) purchased for the project, #1 MinION and #3 QFT-4G test kit were transported to NCCD in 2021 and #4 Inc-jet printer in 2022.
- With the exception of the three items above, the purchased equipment are being stored at the Faculty of Veterinary Medicine, Hokkaido University. However, it is currently bidding for exporters and we plans to deliver all the equipment to UB by March 31, 2023.
- Installation of the BSL-3 facility at the IVM is scheduled to take place by March 31, 2023.