Under project of the

"CONTROL OF TUBERCULOSIS AND GLANDERS"

/SATREPS project/

GLANDERS: Progress activity and Future activity implementation in IVM

Duration: Nov, 2022 to Feb, 2024

21 February, 2024

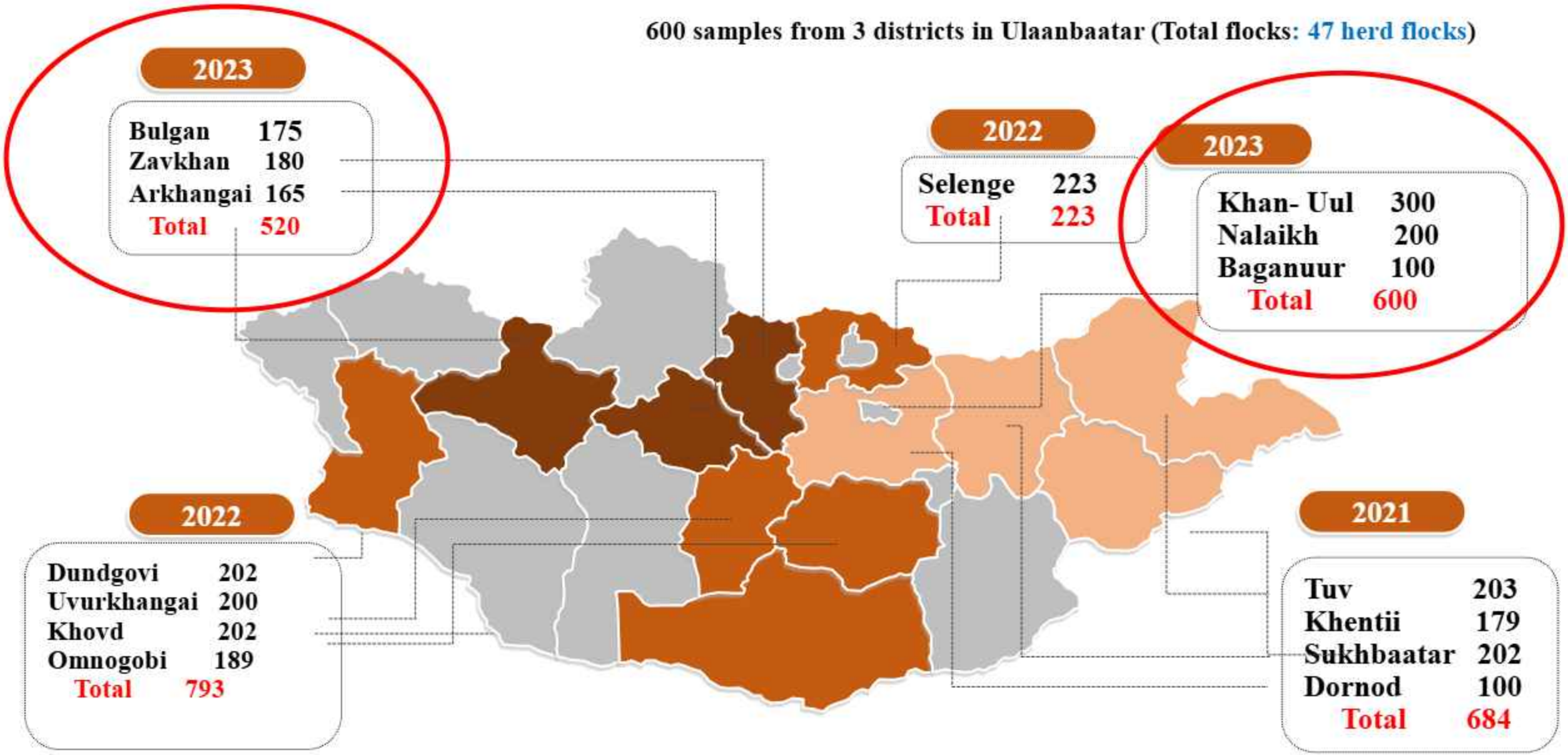
Inputs	Duration
3-3. Molecular-epidemiological and sero-epidemiological evaluation of the epidemics of *B. mallei* infection in horses	
3.3.1 To perform a sero-epidemiological survey on *B. mallei* infection (hislory) by testing the sera obtained from horse herds in the project area with the conventional methods (complement-fixation test) in IVM.	10/2020 - 05/2025
3.3.4 To assess the transmission and distribution of *B. mallei* in horse by performing the comprehensive gene screening using a next-generation sequencer on the isolated strains, which are obtained by culturing specimens of lesioned part of the infected horses	03/2022 - 05/2025

Map of sample collection area

Total: 2820

2220 samples from 43 soums of 12 provinces (Total: 256 herd flocks)

600 samples from 3 districts in Ulaanbaatar (Total flocks: 47 herd flocks)



RESULT OF SERO SURVEILLANCE

Date	Province	Number of samples	Positive by CFT(%)
2021	Tuv	203	1 (0.5)
	Khentii	179	1 (0.6)
	Sukhbaatar	202	9 (4.5)
	Dornod	100	2 (2.0)
2022	Dundgovi	202	3 (1.5)
	Uvurkhangai	200	6 (3.0)
	Selenge	223	3 (1.3)
	Khovd	202	0 (0.0)
	Omnogovi	189	0 (0.0)
2023	Bulgan	175	0 (0.0)
	Zavkhan	180	1 (0.6)
	Arkhangai	165	2 (1.2)
	Baganuur	100	1 (1.0)
	Nalaikh	200	1 (0.5)
	Khan-Uul	300	1 (0.3)
Total		2820	31 (1.1)

Inputs	Duration
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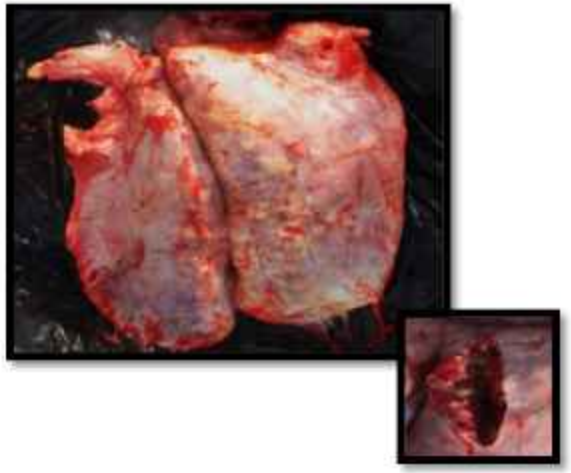
3.3.1 To perform a sero-epidemiological survey on *B. mallei* infection (hisTory) by testing the sera obtained from horse herds in the project area with the conventional methods (complement-fixation and plate agglutination) in IVM.	10/2020 - 05/2025
3.3.4 To assess the transmission and distribution of *B. mallei* in horse by performing the comprehensive gene screening using a next-generation sequencer on the isolated strains, which are obtained by culturing specimens of lesioned part of the infected horses	03/2022 - 05/2025

Clinical Symptoms of Glanderous horses/results of ST

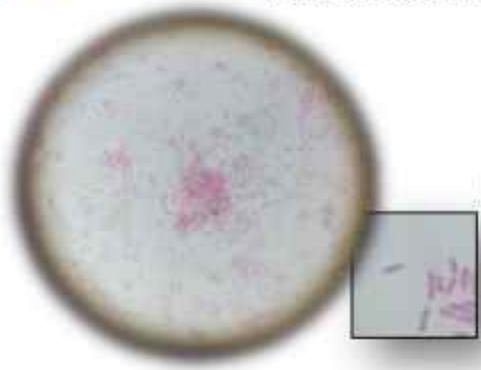
№	Name	**skin thickness** (mm)			Edema diameter (mm)		Result
		Before. inj	24 hrs	48 hrs	24 hrs	48 hrs	
1	23001	3	25	18	35	33	Positive
2	23002	3	23	50	30	90	Positive
3	23003	3	27	17	30	32	Positive

Result of isolation and identification for *B.mallei*

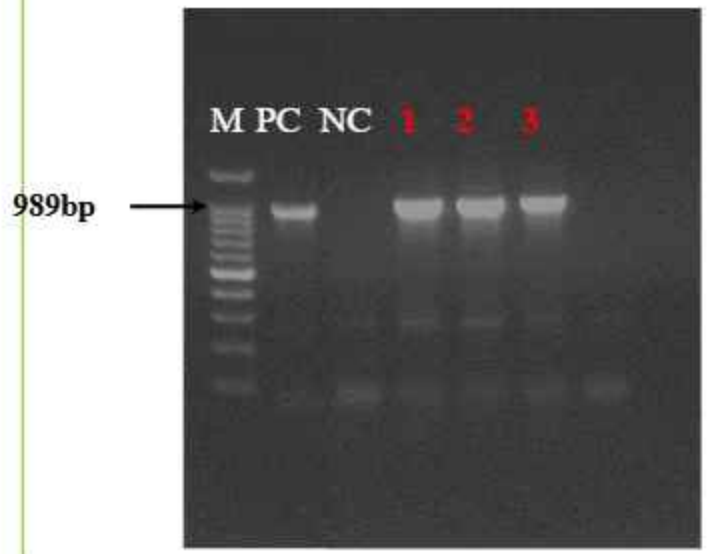


Isolation

Identification



Gram staining



Result of standard PCR with species specific primers of *B. mallei* in **2023**

M - 100bp DNA ladder,
PC - positive control,
NC - negative control
1 - 3 new isolates

PCR detection of *B. mallei* using primer pairs
Bma-ISO407-flipF (5"TCA-GGT-TTG-TAT-GTC-GCT-CGG-3")
Bma-ISO407-flipR (5"CTA-GGT-GAA-GCT-CTG-CGC-GAG-3")

Outputs 3.3.4

Table 4. Collected samples from slaughterhouses and stakeholder in 2020 to 2024

№	Livestock species	Samples (lung. spleen, testis, kidney, liver and skin)	Bacteriology	Confirmed by PCR			Date of Isolated
				Burkholderia spp	B. mallei		
1	Horse[1]	33	33	2		-	2021-2023
	Camel[1]	7	7	2		-	2022
2	Horse[2]	42	76	21	8	n=1	2018
						n=2	2019
						n=4	2022
						n=1	2023
TOTAL		**82**	**116**	**25**		**8**	

Bacteriology was performed under the "**OIE Terrestrial Manual" used as a guideline.**

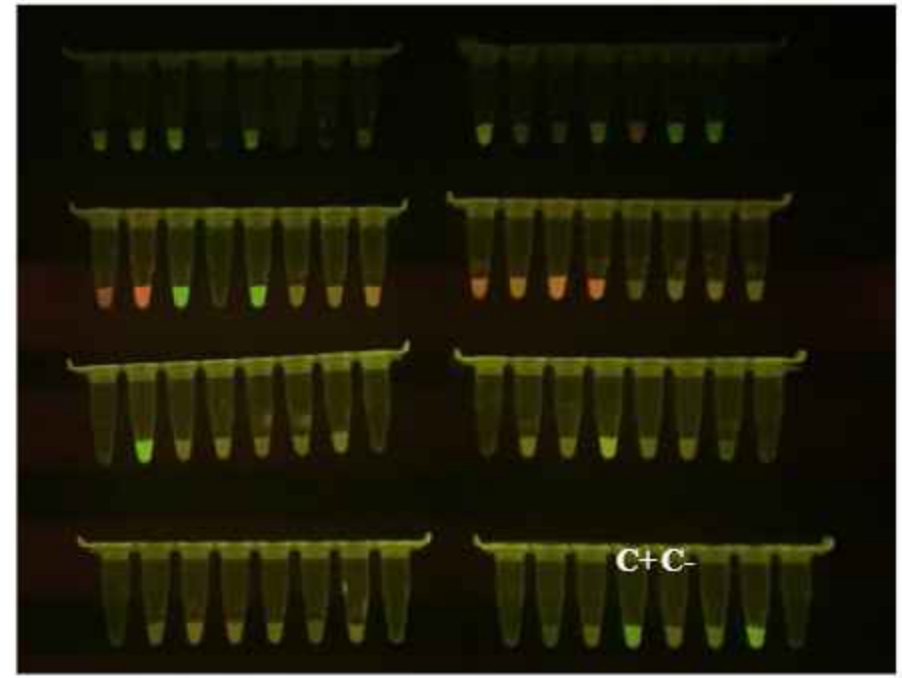
[1]Samples collected from slaughtered horses in SLAUGHTERHOUSES

[2]Samples collected from Glanderous horse that were EUTHANIZED

Comparative of diagnostic tests result for the detection of Glanders, including dry LAMP-PCR

Totally: 47
47 swabs (*nasal, eye, skin*) from suspected horses & others

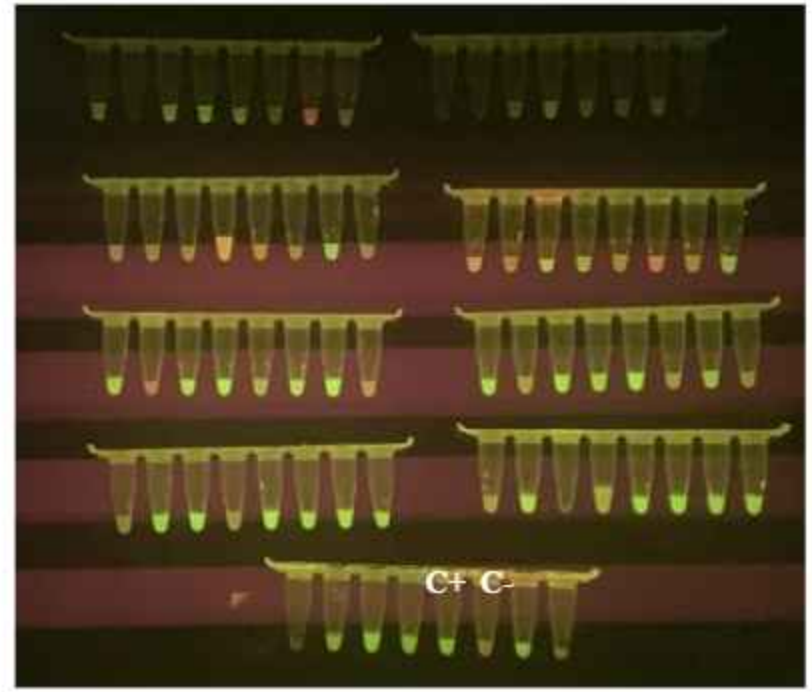
№	Province	LAMP-PCR result positive/total tested	bacteriology	skin test	CFT	iELISA
1	Tuv	1/8 (12.5)	bacteriology is ongoing, result is not finalized	+	+	+
2	Khentii	1/13 (7.6)		NT	+	-
3	Arkhangai	0/4 (0)		NT	-	-
4	Bulgan	1/21 (4.7)		NT	-	NT
5	Ulaanbaatar	1/1 (100.0)		NT	-	NT
	Total	4/47 (8.5)		1		



Comparative of diagnostic tests result for the detection of Glanders, including new candidate test

Totally: 11
11 tissue samples *(lung, liver, spleen, skin node, testis)* from Glanderous/suspected horses

Horse ID	Province	Status				
		LAMP-PCR		Bacteriology	CFT	Skin test
		Nasal swab	Tissue			
Do1-23	Dornod	-	+	-	-	-
Do2_23	Dornod	+	+	-	+	+
Tuv3_22	Tuv	+	+	+	+	+
Tuv4_m_22	Tuv (1)	+	+	+	+	+
Tuv5_22	Tuv	-	+	-	+	+
Tuv6_f_22	Tuv (1)	-	+	-	-	-
Tuv7_22	Tuv	-	+	-	-	+
UB23001	UB	+	+	+	+	+
UB23002	UB	+	+	-	+	+
UB23003	UB	+	+	-	+	+
Um2209	Umnugobi	NT*	+	-	NT*	NT
		66%	100 %	27%		



* - no serum/no swab

(1) - mare and her foal

Inputs

4.3. Risk assessment of *B. mallei* infection as a zoonotic disease

4.3.1. To determine a study design (e.g., the preparation of survey procedures, the unification of analytical methods and so on) in order to perform the risk assessment associated with *B. mallei* transmission between livestock and human, through the discussions between medical and veterinary glanders research groups.

1. The collected questionnaires (*for the RA study of Glanders)* from **150** herders of **47** soums in **12** provinces
2. The collected questionnaires **14 slaughterhouses near UB** (located in Emeelt and Nalaikh)
3. Case control - Prepared to information sheet from IVM to NCCD

Bovine Tuberculosis (bTB)

Tables show the Operation plan of the project (Revised)

Outputs 3.1.1

Collected samples from Slaughterhouses in UB /Emeelt and Nalaikh/				
Lung samples	**Cattle/yak**	**Sheep**	**Camel**	**Total**
2022	**87**	**77**	**21**	**185**

Bacteriology was performed under the "OIE Terrestrial Manual of Standard for Diagnostic Tests and Vaccines" used as a guideline.

3-1. Molecular-epidemiological evaluation of the epidemics of *M. bovis* Infection in livestock
3.1.1. To isolate tuberculosis complex using the L-J mediua in IVM, from the tubercles-suspected granulomas samples collected from cattle and sheep at slaughterhouses and meat markets in the project target areas.
3.1.2. To estimate the prevalence of *M. bovis* in the tuberculosis complex isolated from cattle and sheep by determining the presence of *M. bovis* in the colonies grown on L-J medium using the genetic detection method developed in the Activity 1.1.
3.1.3. To investigate the endemic status of *M. bovis* infection including subclinical infection by performing the Interferon-Gamma Release Assay (IGRA) on herds in which *M. bovis*-detected cattle were kept.

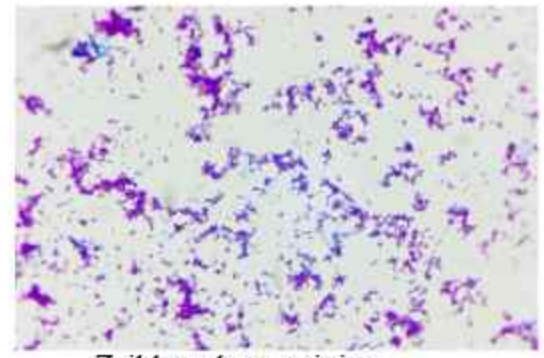
Bacteriology

Milk samples from some province in 2022 including from UB in 2023

Date	Place	Milk/samples
2022	Selenge	22
2023	Tuv Bayanchandmani	23
	TOCA market	3
	Tuv Batsumber	14
	UB songino khairkhan district	82
Total		**144**

Total 144 milk samples were processed then cultured in Lowenstein media incubating at 37°C in for up to 4-10 weeks. Mycobacterium like colony growth was observed in the 12 samples.

Colonies growth on LJ medium



Zeihl-neelsen staining

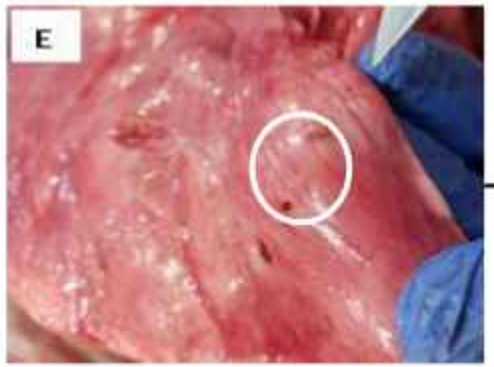
M-marker 100bp, NC negative control, PC-BCG positive control, from NCCD positive control, 1-12 samples

We performed PCR to detect M. tuberculosis on 12 suspected colonies.

However, our PCR did not yield any positive results.
Subsequently, we forwarded these all colonies to the NCCD for confirmation but no positive results were obtained.

Bacteriology results

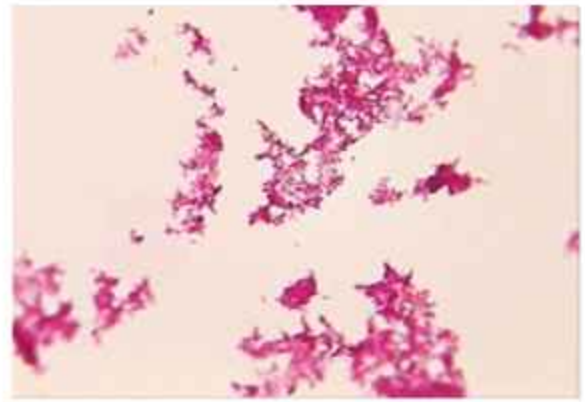
The lung samples were processed then cultured in Lowenstein media incubating at 37°C in for up to 8-10 weeks. Mycobacterium like colony growth was observed in the sample “Y1” and “Sh17”.



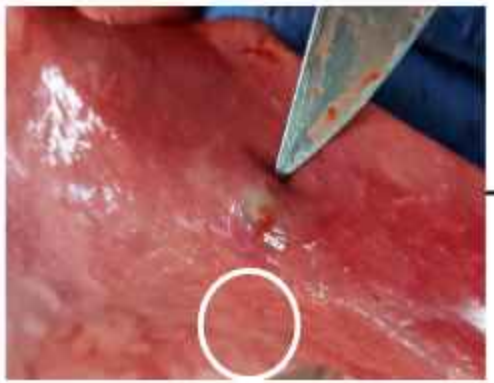
Cattle Lung “Y1”

Lowenstein
Jensen media

multiple colony observed

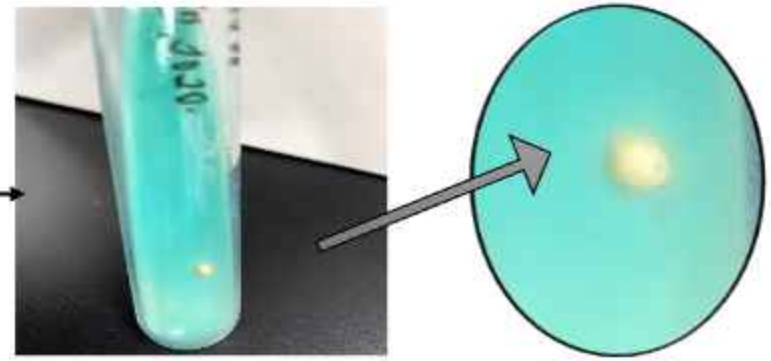


Zeihl-neelsen staining

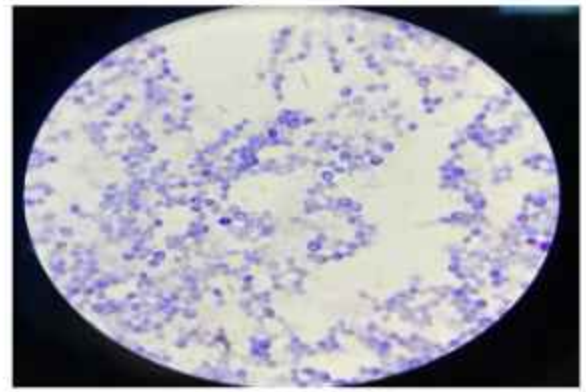


Sheep Lung

Lowenstein
Jensen media

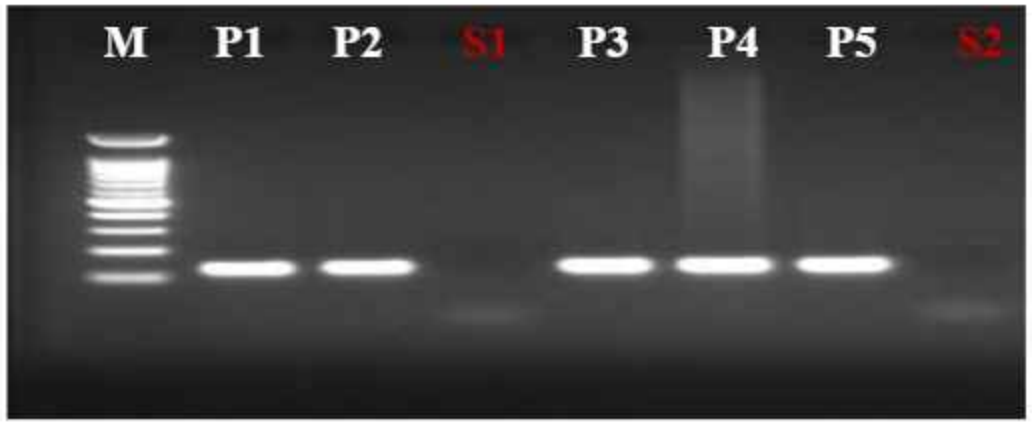


Single colony observed



Zeihl-neelsen staining

Result (Molecular biology)



M - 100 bp molecular weight marker
P1 - positive control M.tuberculosis
P2 - positive control M. bovis
S1- lung sample Y1
P3 - positive control BCG
P4 - positive control from NCCD
P5 - positive control from NCCD
S2- lung sample SH17

Protocol:

- ✓ DNA was isolated from a suspected colony by a purification kit and measured its concentration at 260/280 nm
- ✓ PCR mixer mix was prepared using MTBC specific primer (IS6110) PCR conditions were as below

  94°C for 2 min

  94°C for 30 sec

  63°C for 30 sec

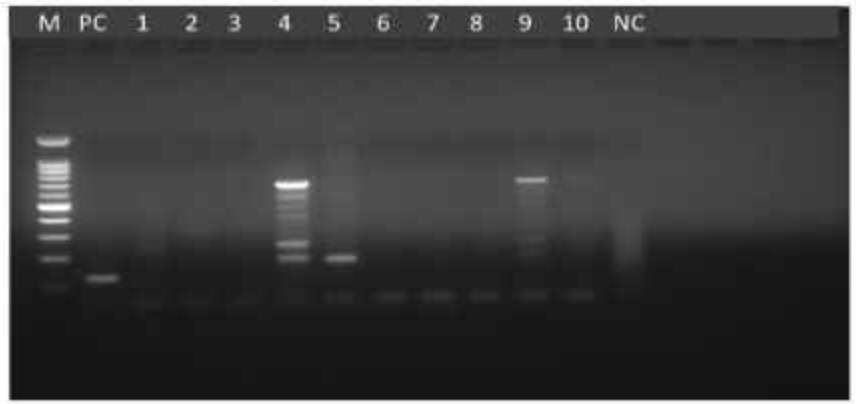
  68°C for 1 min

  4°C with total of 35 cycles
- ✓ PCR products were run at 100V for 25 mins in 1.5% agarose gel

Molecular biology results 2023

We conducted direct PCR /***M. tuberculosis* and *M. bovis***/ to 20 **milk** samples, but there is **no positive** reaction.

IS6110 sequence



Distinguishing ***M.bovis*** from ***M.tuberculosis***

Based on ***pncA, oxyR*** sequence
M - 100 bp molecular weight marker
PC- Positive control from NCCD
S1- S4- **Milk samples**
PC- Positive control BCG
NC- Negative control

Mycobacetrium spp like bacteria's DNA sequence, analysis

performed at the RCZC, Japan

	original ID	Closest species	identity	rpoB primer pair can detect Mycobacteria and Streptomy	Overlapped peak means mixed culture	
N	DDW	Arthrobacter crystallopoietes		Contamination of lab reagents?	overlapped peak: 1	
1K	2 (?)	Arthrobacter crystallopoietes		Contamination of lab reagents?	overlapped peak: several	
2B	4-1 ?, y-1 ?	Mycolicibacter hiberniae	227/228	May be *Mycolicibacter hiberniae*, environmental bacteria	almost no overlapped peaks	GGTGGTC
2K	4-1 ?	Mycobacterium nonchromogenicum	224/230	Novel species, environmental bacteria	very good	CCGGTGC
3B	y-1-1?, 4-1-1?	Mycolicibacter hiberniae	229/230	May be *Mycolicibacter hiberniae*, environmental bacteria	overlapped peak: more than sever	CCGGTGC
3K	y-1 (1-1)	Mycobacterium nonchromogenicum	224/230	Novel species, environmental bacteria	very good	CCGGTGC
4K	5	Mycobacterium nonchromogenicum	225/231	Novel species, environmental bacteria	very good	CCCGGTC
5B	Arg7	Arthrobacter crystallopoietes		Contamination of lab reagents?	overlapped peak: several	
5K	Arg * ?	Mycobacterium nonchromogenicum	225/231	Novel species, environmental bacteria	very good	CCCGGTC
6B	Arg2 ?	Mycobacterium nonchromogenicum	225/231	Novel species, environmental bacteria	very good	CCCGGTC
6K	Arg ** ?	Arthrobacter crystallopoietes		Contamination of lab reagents?	overlapped peak: a little more than several	
7B	Arg3	Arthrobacter crystallopoietes		Contamination of lab reagents?	overlapped peak: more than several	
7K	Arg *** ?	Corynebacterium provencense	228/231	Not *Mycobacterium species*	several small overlapped peaks	CCGGTCT
8B	SebR171? 191	Mycolicibacterium confluentis or Mycobacterium porcinum	211/216	Novel species, environmental bacteria	overlapped peak: several	
8K	SEBR 171	Mycobacterium nonchromogenicum, M. virginiensis or M. hiberniae	222/228		almost no overlapped peaks	GGTGGTC
9B	Bn 3 ?	Arthrobacter crystallopoietes		Contamination of lab reagents?	overlapped peak: a little more than several	
9K	BN 3	Mycobacterium nonchromogenicum	197/202	Novel species, environmental bacteria	almost no overlapped peaks	TCGGCAC
10B	BN 7	Mycobacterium sp	227/232	Mycolicibacterium poriferae 226/232, Mycobacterium chubuens	overlapped peak: several	
10K	BN 7	Mycobacterium nonchromogenicum	224/230	Novel species, environmental bacteria	very good	CCGGTGC
11B	373070	Arthrobacter crystallopoietes		Not *Mycobacterium species*	heavy overlap, or NG seq	
11K	373070	Streptomyces tsukubensis	184/195	Not *Mycobacterium species*	overlapped peak: a little more than several	
12B	373403	Mycobacterium nonchromogenicum or Mycolicibacter virginiensis or M. hi	204/210		almost no overlapped peaks	
12K	373401 ?	Arthrobacter crystallopoietes	205/218	Not *Mycobacterium species*	overlapped peak: more than several	
13B	373062	Mycobacterium bolletii or Mycolicibacterium phocaicum	220/226	Novel species, environmental bacteria	almost no overlapped peaks	TGGCGGC
13K	373062 ?	Comamonas thiooxydans	207/213	Not *Mycobacterium species*	heavy overlap	

2B, 3B: Two similar size band by IS6110PCR

5K, 10K: Faint band by IS6110PCR (Size was different from above)

Mycobacterium nonchromogenicum Mycobacterium other species B-boiled K-kit

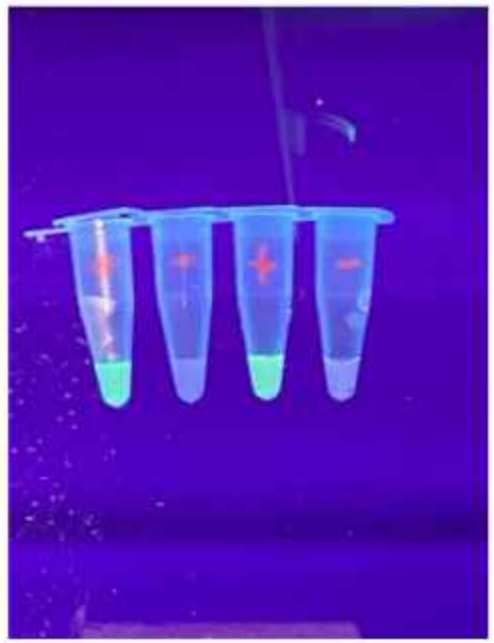
Nested PCR –Negative

Only two isolates, which yielded positive results in molecular biology tests, exhibited a 99.5% similarity to the ***Mycolicibacter hyberniae*** species.

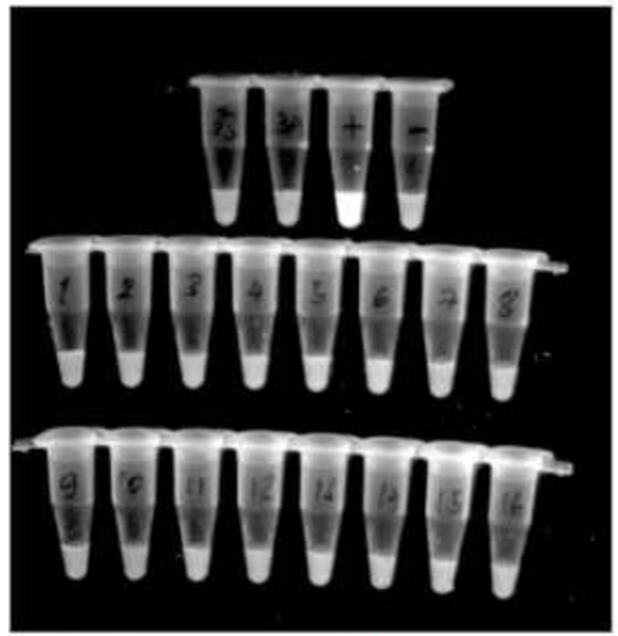
Additionally, According to the DNA sequencing results six isolates belonged to the new ***Mycobacteria*** **genus**.

Result of the dry LAMP-PCR for diagnosis of bTB

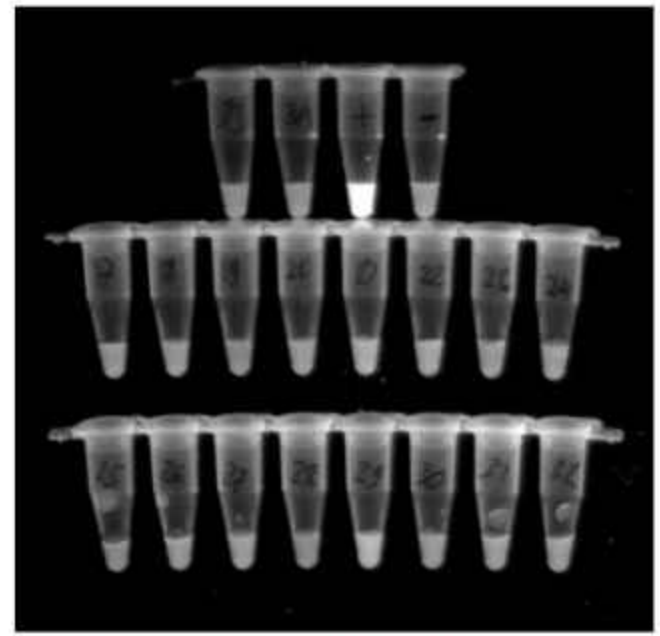
In 2023, DNAs was extracted from 34 lung samples collected near UB City at a slaughterhouse using the Proteinase K protocol. Subsequently, the extracted DNA was analyzed through a dry LAMP reaction."



+ Positive control BCG DNA
- Negative control Water



+ Positive control BCG DNA
- Negative control Water
1-16 Lung DNA samples
33-34 Lung DNA samples



+ Positive control BCG DNA
- Negative control Water
17-34 Lung DNA samples

Result: all tested samples were negative.

Tables show the Operation plan of the project

Outputs 3.1.3

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SEROLOGY RESULTS in UB, 2023 by IDVet ELISA

N	District	Stimulated plasma samples	Plasma sample induced with PPD_B		Plasma sample induced with PPD_A		Skin test /skin thickness increased >4mm considered positive/			
			IGRA assay		IGRA assay		On the left side /M. bovis PPD/		On the right side /M. avium PPD/	
			Tested	Positive	Tested	Positive	Tested	Positive	Tested	Positive
1	Songino khairkhan	290	248	16	248	24	290	30	160	26
2	Khan-Uul	170	140	3	140	2	170	0	124	0
3	Bayanzurkh	40	0	0	0	0	40	0	10	0
	Total	500	388	19	388	26	500	30	294	26

Bovine Tuberculosis (bTB)

Johne's disease surveillance /serum samples/

№	District	Johne's Disease screening ELISA	
		Tested	Positive
1	Songinokhairkhan	255	2
2	Khan-Uul	37	1
3	Bayanzurkh	0	0
	Total	292	3 (1.02%)

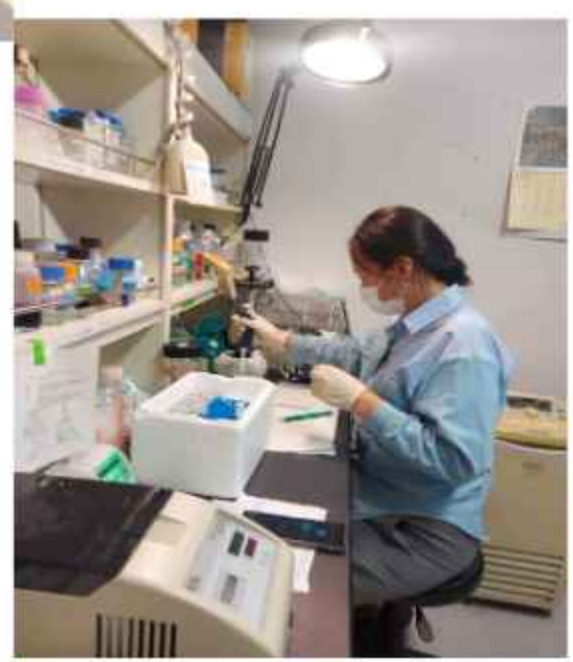
ID number	Johne's Disease screening ELISA Positive samples		Plasma sample induced with PPD_B		Plasma sample induced with PPD_A	
			IGRA assay			
	Aver_ S/P	IFN_ status	Aver S/P	IFN_ status	Aver S/P	IFN_ status
SHB-7	0.384	P	9,2	P	32.27	P
SHB-236	0.69	P	369.5	P	218.4	P
KUB-12.1	0.672	P	6.2	N	39.2	P

Sample collected from cattle farms near UB

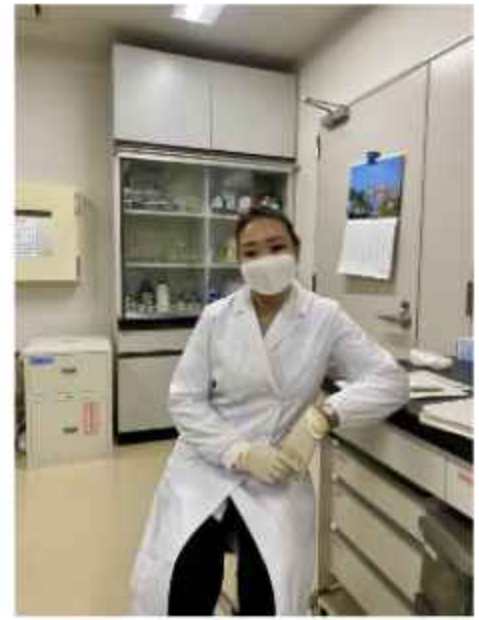
Trained at Hokkaido University (Dec 2022 to Feb, 2024)

Associate-Researcher
Dr. Lkham
Trained on Dec 2023

Associate-Researcher
Dr. Khurtsbaatar
Trained on Jan 2023



Associate-Researcher
Dr. Enkhtuul
Trained on Dec 2022



Associate-Researcher
Dr. Enkhtsetseg
Trained on Dec 2022

Trained at Hokkaido University on Dec, 2023

Associate-Researcher
O. Khurtsbaatar

Associate-Researcher
Ts. Batbold

Associate-Researcher
G. Ulziisaikhan

Assistant-Researcher
T. Agiimaa

Goal in 2024

- To perform bTB surveillance of flocks and slaughterhouses near UB.
- To perform bio-sample collection from Glanderous horses.
- To perform Johne's disease surveillance flocks near UB
- To isolate of the *B. mallei* field strains,
- To cooperate with HU (to perform evaluation/validation of the new rapid candidate test /dry LAMP-PCR, ICT, iELISA, etc/)
- To write scientific papers
- To send trainees to Japan