ATTACHMENT 2

United States Department of Agriculture
Animal and Plant Health Inspection Service

National Veterinary Services Laboratories
1920 Dayton Ave., Ames, IA 50010
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***Mycobacterium tuberculosis* Final Genotyping Report**

February 16, 2012

This is a genotyping report for a *M. tuberculosis* isolate (NVSL: Accession No. 12-001668, TB No. 12-02772) obtained in February, 2012 from necropsied lung tissue from Sabu (aka 'Look Chai'), a deceased Asian male elephant. Sabu (b. 1982) was diagnosed with acute arthritis and euthanized on January 11, 2012, in his stall at the Performing Animal Welfare Society's ARK 2000 sanctuary in San Andreas.

M. tuberculosis had been cultured in December 1999 at the NVSL from a trunk wash from Sabu collected at the Ringling Bros. facility in Williston, FL in November, 1999. Accordingly, PAWS and the UC Davis necropsy team excised lung tissue and forwarded it to the Mycobacteria and Brucella Section of the National Veterinary Services Laboratory, where it was received on January 17, 2012; culture of the lung tissue was initiated on January 18. Acid-fast bacteria were observed in a liquid media culture on January 31 and subjected to genotyping the weeks of February 5 and February 12.

[In addition to the lung tissue, swabs of lung tissue were obtained by Jackie Gai, of PAWS, and received at the NVSL on January 13; as of the time of writing, acid-fast bacilli have been observed in the associated liquid and solid media cultures and are scheduled for genotyping later this month.]

Two techniques, widely used by laboratories working on *M. tuberculosis*, were used to genotype the isolate. The first method is spoligotyping, and relies on the presence or absence of so-called 'direct repeats' in the *M. tuberculosis* genome to differentiate between isolates. It is considered a 'medium resolution' genotyping technique. The second method is variable number tandem repeat (VNTR), and this examines the genome of a given *M. tuberculosis* isolate for the presence of 'tandem repeats'. Differentiation between isolates can be mediated by differences in the numbers of tandem repeats observed at 11 or more loci in the genome. The VNTR method is considered to be a 'high resolution' genotyping technique.

The NVSL used a VNTR assay targeting 11 loci ('VNTR-11') from 2009 – October 2011. In November 2011 the NVSL adopted an expanded version of the VNTR method, targeting 24 loci ('VNTR-24'); this expanded panel is equivalent to the one used by the CDC, and its contracting state laboratories in California and Michigan. As of February 2012, at least one *M. tuberculosis* isolate from every positive elephants in the NVSL database has received the upgraded VNTR-24 assay. (For some elephants with multiple isolates, if every isolate shares the same spoligotype and VNTR-11 profiles, then only one of these isolates has been upgraded to VNTR-24).

Spoligotype and VNTR-24 profiles for the February 2012 isolate of *M. tuberculosis* from Sabu were compared to profiles for all elephant isolates in the NVSL BioNumerics database, including the isolate made from Sabu's trunk wash from November 1999.

The spoligotype for the *M. tuberculosis* strain from Sabu's lung tissue possessed octal code 407777777760771. This is different from that observed for the *M. tuberculosis* strain recovered from Sabu's trunk wash in 1999 (octal code 477777777760731). To confirm that this observation was reproducible, the culture from Sabu from 1999 / 2000 was retrieved from the freezer, re-extracted, and subjected to spoligotyping the week of February 12, 2012; it generated the same spoligotype that was observed originally, ie, 477777777760731.

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The spoligotype generated from the February 2012 Sabu isolate was identical to the spoligotype observed for isolates from one other elephant in the NVSL database, Tb Nos. 99-3867 and 00-3206 (Figure 1, below). During 1999 this elephant was a herd mate to Sabu.

The VNTR-11 and (where present) VNTR-24 profiles for the *M. tuberculosis* isolates from Sabu were subjected to a statistical analysis using the unweighted pair-group method with arithmetic mean (UPGMA) technique; the resultant dendrogram is shown in Figure 2 (below). The VNTR-11 / VNTR-24 profile for the February 2012 isolate from Sabu is very different from that observed for the 1999 isolate, and in fact segregates into a cluster occupied by the same isolate, TB No. 99-3867, from the elephant which was a herd mate to Sabu.

There is a difference in VNTR-24 profile between the 2012 Sabu isolate and the 1999 herd mate isolate of one repeat, at one locus (VNTR 2163 aka QUB11b); this may reflect a minor, host-related genetic difference in this lineage of *M. tuberculosis*.[1]

Taken together, the spoligotype and VNTR profiles for the February 2012 isolate from Sabu indicate that this elephant was infected with a second strain of *M. tuberculosis*. This second strain possesses a genotype (spoligotype + VNTR profile) identical to that recorded for an isolate (TB No. 99-3867) made in 1999 from a herd mate to Sabu. This herd mate was euthanized in October of 1999 due to arthritis and tuberculosis.

It could be hypothesized that Sabu acquired this second strain from his herd mate prior to the latter's death, but that, at the time of the trunk washing obtained from Sabu in November 1999, bacteria from this second strain either chanced not to be present in the wash, or had not replicated to numbers sufficient to be detected in the wash.

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[As of the writing of this report, the VNTR-24 assay has not yet been subjected to ISO17025 certification by the NVSL.]

[1] Readers are invited to submit the spoligotype (as the octal code) and VNTR data (as 12, or 15, or even 24 loci) for Sabu to the MIRU-VNTR*plus* website, http://www.miru-vntrplus.org/MIRU/index.faces, to learn if any *M. tuberculosis* isolates of human origin share this genotype

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March 14, 2012

NVSL Accession # 11-002174, TB No. 11-2628, 'Rebecca', PAWS, Galt, CA

This is an updated version of a genotyping report originally distributed to VS Staff and Oklahoma State Health Lab staff on February 18, 2011, for a *Mycobacterium tuberculosis* isolate recovered from Rebecca, a 50 year-old Asian elephant, owned by the Performing Animal Welfare Society (PAWS), who died on January 8, 2011. Lung and lymph node specimens were received at the NVSL on January 12, 2011.

The *M. tuberculosis* isolate generated from Rebecca's tissues was subjected to genotyping analysis (i.e., spoligotyping and variable number tandem repeat, or VNTR) during February 2 – 8, 2011.

A prior trunk wash submission from Rebecca, delivered to the NVSL in 2002 (when Rebecca was owned housed at PAWS), generated a *M. tuberculosis* isolate, Accession No. 154146, TB No. 02-2460. This isolate was not subjected to VNTR until March 2011; accordingly, information about the 2002 isolate of Rebecca was not included in the original genotyping report for her February, 2011 isolate. Accordingly, information about the 2002 isolate is included in this updated genotyping report.

Because the spoligotype profile for the 2002 isolate from Rebecca (performed in 2005) was faint, another aliquot of DNA – extracted in the Fall of 2011 for the purpose of obtaining an expanded VNTR-24 panel for the 2002 isolate– was subjected to spoligotyping the week of February 19, 2012. A more satisfactory spoligotype (albeit one with the same profile as that performed in 2005, i.e., octal code 000000007760771) was observed, and is used in this report.

The 2002 and 2011 isolates differ in both their spoligotype (octal code 000000007760771 Vs 776377774020771) and VNTR profiles, indicating that Rebecca was infected with two different strains of *M. tuberculosis* (Figure 1, below). Neither spoligotype has previously been observed in the NVSL database, which makes it unlikely that these isolates represent contaminants that have been circulating in the laboratory.

The spoligotypes for both isolates has previously been recorded at the online, open-access SITVIT and MIRU-VNT*plus* websites: the 2002 isolate is Shared Type (ST) 4 (171 matches with SITVIT entries), while the 2011 isolate, ST383 (8 matches with SIVIT entries). Neither isolate's VNTR-24 profile is recorded in either the SITVIT, or MIRU-VNT*plus*, websites. Lauren Cowan at the CDC kindly agreed to a request from the NVSL to see what other isolates in the CDC's proprietary internal database matched the spoligotype for the 2011 Rebecca isolate. Cowan indicated that the spoligotype for the 2011 isolate had characteristics of the EuroAmerican S and EuroAmerican Haarlem *M. tuberculosis* lineages.[1] However, none of the *M. tuberculosis* isolates in the CDC internal database possessed a spoligotype matching that for the 2011 isolate from Rebecca.

When compared with the 186 Mycobacterium strains in the MIRU-VNT*plus* website, the 2002 Rebecca isolate segregated into a cluster with a 1999 (human) isolate from Uganda (No. 2224). The 2011 Rebecca isolate segregated into a larger cluster shared with (human) isolates, of the 'Haarlem' lineage of *M. tuberculosis*, from human patients in the interval 2002 – 2003 (see the accompanying pdf file of the MIRU-VNT*plus* dendrogram containing the two Rebecca isolates, highlighted in yellow).

A comparison of the two Rebecca isolates with all other elephant *M. tuberculosis* isolates (for which complete VNTR-24 profiles are available) in the NVSL database is presented in Figure 2. This analysis was generated using the Unweighted Pair Group

[1] www.biomedcentral.com/1471-2180/6/23

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Method with Arithmetic Mean (UPGMA) algorithm from VNTR-24 profiles. A dendrogram, presented on the left side of the VNTR number matrix, demonstrates the clustering of identical and / or closely related isolates. Based on the dendrogram, the two Rebecca isolates, and the two isolates from Sabu, another former Ringling Bros. elephant later housed at PAWS / ARK 2000, have no similarity to each other.

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Figure 1. Comparison of genotypes for 2002 (TB No. 02-2460) and 2011 (TB No. 11-2528) isolates of *M. tuberculosis* from Rebecca

Spoligotyping	VNTR																												
	VNTR 0424	VNTR 0577	VNTR 1644 (MIRU16)	VNTR 1955	VNTR 2165 (ETR A)	VNTR 2401	VNTR 2461	VNTR 2687 (MIRU24)	VNTR 2996 (MIRU26)	VNTR 3192 (MIRU31)	VNTR 4052 (QUB-26)	VNTR 3007 (MIRU27)	VNTR 0580 (MIRU04)	VNTR 0802 (MIRU40)	VNTR 0960 (MIRU10)	VNTR 2163b (QUB-11b)	VNTR 3690	VNTR 4156	VNTR 0154 (MIRU02)	VNTR 2059 (MIRU20)	VNTR 2347	VNTR 2531 (MIRU23)	VNTR 3171	VNTR 4348 (MIRU39)	TB No	Spoligo Octal Code
	3	4	3	1	4	2	1	1	5	3	1	3	3	4	3	4	4	1	1	2	3	5	3	2	02-2460	000000007760771
	2	3	2	3	3	4	2	1	5	4	10	3	2	3	5	3	3	3	2	2	4	5	2	2	11-2528	776377774020771

Animal Care Elephant TB StatPak Official Annual Test

License # 93-C-0074
Customer # 3551
Name of Facility PAWS
Site Name 002 - San Andreas
Date 6/28/11

Signature Federal Vet [redacted]
Printed Name of Federal Vet Pamela Smith

Signature of Attending Vet
Printed Name of Attending ve

Blood draw 6/27/11 (except Lola - 6/28/11) Tests run 6/28/11 (Stat Paks)

	Animal	Photo taken	Kit # Serial	Kit # Lot	Results Non-reactive	Results Reactive	Results Inconclusive	Comments	MAPIA Results 7/5/11	Initials
1	Mara	Previously	TBV10SID	Diluent TBSD0915id	*	—	—	African ♀ 31 yrs.	—	
2	Maggie				*	—	—	African ♀ 31 yrs.	—	
3	Annie					*		Asian ♀ 51 yrs	Reactive	[redacted]
4	Gypsy					*		Asian ♀ 44 yrs	Reactive	[redacted]
5	Wanda				*	—	—	Asian ♀ 52 yrs	—	

FOIA 12-03299

Elephant TB StatPak Official Annual Test

License # 93-C-0074
Customer # 3551
Name of Facility Site-002 - San Andreas
Site Name PAWS
Date 6/27/11

Signature Federal Vet (b)(6)
Printed Name of Federal Vet Ronald Smith

Signature of Attending Vet
Printed Name of Attending ve (b)(6)

	Animal	Photo taken	Kit # Serial	Kit # Lot	Results Negative	Results Positive	Results Inconclusive	Comments	NAPTA Results	Initials
(6)	Nick	Previously	TBU10016	Dilaent TB5D09151B	*			Asian ♂ 18 yrs.		
(7)	Sabu	Yes	↓	↓		*		Asian ♂ 29 yrs.	Reactive	(b)(6)
(8)	Lulu	Previously	↓	↓	*			African ♀ 45 yrs		

FOIA 12-0B289 99