The Pan-SL-CoV/GD sequences may be from contamination.

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ABSTRACT

Recently, There were much hype about an alleged SARS-like coronavirus being found in samples of Malayan pangolins (Manis Javanica) possessing nearly identical RBD to the SARS-CoV-2 coronavirus. Prominent journals cite the alleged discovery to claim that pangolins may be one of a possible intermediate host for the zoonotic transmission of SARS-CoV-2 to humans.

Here, we report that all databases used to support such a claim, upon which metagenomic analysis was possible, contained unexpected reads and was in serious risk of contamination. Here we also report that the presence of unexpected reads are directly related to the presence of coronavirus reads. Finally, we deduced the actual causative agent of the death of the pangolins sampled in GuangDong 2019 where the claim of coronavirus detections was made.

METHODS

The NCBI Trace tool

The NCBI SRA archive come with it's own tool called Trace, which identifies the origin or reads within the SRA dataset through the recognition of unique K-mers within the nucleotide sequence. Multiple reads of 32 nucleotides is taken from each read to identify the reads toward an origin by comparison with a large database of reference sequences, which produces a classification signal. Then read of 64 nucleotides are taken from each of the read for definitive mapping toward species in the reference database. If any one of the 32nt or 64nt K-mers are found in more than one reference sequence, the reads are instead classified at the lowest phylogenetic classification node where reference sequences containing such a K-mer is found.

The 32nt TRACE generate a "strong signal" classification of sequence origin useful for the deduction of the content of the sample by organism of origin, accessed via the NCBI Krona charting tool,

While the 64nt TRACE generate a definitive classification signal used for the exact tracing of reads to the origin from a specific Species/Taxon, used for the exact classification of reads.

Both the 32nt and 64nt TRACE analysis classify their reads according to the lowest common taxonomical node where K-mers from said read are present in the reference sequence database, a strategy known as "lowest non-ambiguous mapping". Such a strategy avoids the problem with RNA degradation or sequencing errors by excluding potential errors in reads, without introducing potential ambiguous classification by clustering ambiguous reads under the lowest common classification node such ambiguity is found.

Therefore, if TRACE gives an identification to a specific taxonomical node for a sequence read, it could be from any of the taxonomical nodes and species classified under the node, but it could not be from a taxonomical node or species that is not under said node. E.g. if TRACE says hominoidea which was classified under Catarrhini; Simiiformes; Haplorrhini; Primates; Euarchontoglires, Then it can't be from a pangolin since pangolins (Manis Spp.) are classified under Pholidota; Laurasiatheria. The lowest common classification node between Primates and Pangolins is Boreoeutheria—reads from parts of the genomes shared between Primates and Pangolins will only be classified to Boreoeutheria, but not further classified down toward either Laurasiatheria or Euarchontoglires. And definitely will not be classified individually toward Pholidota or Primates, or any child nodes or phylogenetic nodes under them.

Specific BLAST analysis

Whenever a genus or species is provided by analysis, a specific BLAST analysis is performed to confirm the presence of reads toward the exact species by a search of the database in question with representative reference sequences of the specific species in question in look for matches that is either: 100% match, or: contained no 100% matches on BLAST when queried against the Pangolin reference sequences available on GanBank.

RESULTS

The Accession numbers and contents of all Pan-SL-CoV/GD related sequencing experiments are listed under the following table.

Table 1: List of available GD Pangolin sample datasets as provided under NCBI GenBank. By Accession number, size and citation by thesis (if claimed to have SARS-CoV-2 related reads by paper).

Associan number	Cizo	SARS Col/ 2 like Coronavirus			
Accession number	Size	SARS-CoV-2-like Coronavirus			
		Identified and Cited?			
SRX6893158	16,491,648				
SRX6893157	9,275,501	Lung12 [3] SRR10168374			
SRX6893156	22,220,187	Lung11 [1]			
<u>SRX6893155</u>	18,067,615	Lung09 [1] [3] SRR10168376			
SRX6893154	16,414,925	Lung08 [1] [3] [4]			
		SRR10168377			
SRX6893153	19,045,923	Lung07 [1] [3] [4]			
		SRR10168378			
SRX6893152	13,527,964				
<u>SRX6893151</u>	16,068,654				
SRX6893150	12,967,281				
SRX6893149	12,590,769				
<u>SRX6893148</u>	15,273,939				

SRX6893147	15,975,904	
<u>SRX6893146</u>	19,038,817	
<u>SRX6893145</u>	19,055,973	
SRX6893144	15,350,468	
SRX6893143	11,527,782	
SRX6893142	20,045,443	
SRX6893141	18,903,834	
SRX6893140	19,986,780	
<u>SRX6893139</u>	39,738,679	Lung02 [3] SRR10168392
<u>SRX6893138</u>	22,900,426	
<u>SRX7756769</u>	107,267,359 PRJNA607174**	M1[2]***
<u>SRX7756766</u>	273,651,431 PRJNA607174**	
<u>SRX7756765</u>	196,761,202 PRJNA607174**	
<u>SRX7756764</u>	222,286,763 PRJNA607174**	
<u>SRX7756763</u>	212,161,250 PRJNA607174**	
<u>SRX7756762</u>	232,433,120 PRJNA607174**	M6[2]***
<u>SRX7756761</u>	113,900,941 PRJNA607174**	
<u>SRX7732094</u>	2,633*	"P2S"[3]

^{*: &}quot;Design: This dataset contains coronavirus-like sequence reads, based on BLAST search."

Article

Extended Data Table 3 | Identification of SARSr-CoV sequence reads in metagenomes from the lung of pangolins using the SARS-CoV-2 sequence (GenBank accession No. MN908947) as the reference

le ID	Animal species	Total reads*	No. mappe	d
1 N	Malayan pangolin	107,267,359	496 ←	
2 N	falayan pangolin	38,091,846	302	
3 N	falayan pangolin	79,477,358	14	
4 N	falayan pangolin	32,829,850	1,100	Not available
5 N	Malayan pangolin	547,302,862	56	
6 N	Malayan pangolin	232,433,120	10	←SRX7756762 "pangolin 2"
B N	Malayan pangolin	44,440,374	12	
0 N	falayan pangolin	227,801,882	0	Not available
. С	hinese pangolin	444,573,526	0	
	N N N N N N N N N N N N N N N N N N N	Malayan pangolin Malayan pangolin	Malayan pangolin 107,267,359 Malayan pangolin 38,091,846 Malayan pangolin 79,477,358 Malayan pangolin 32,829,850 Malayan pangolin 547,302,862 Malayan pangolin 232,433,120 Malayan pangolin 44,440,374 Malayan pangolin 227,801,882	Malayan pangolin 107,267,359 496 ← Malayan pangolin 38,091,846 302 Malayan pangolin 79,477,358 14 Malayan pangolin 32,829,850 1,100 Malayan pangolin 547,302,862 56 Malayan pangolin 232,433,120 10 Malayan pangolin 44,440,374 12 Malayan pangolin 227,801,882 0

Fig.1 the "Extended Data Table 3" of [2]. SRA datasets identified in the available database is pointed out by an arrow, while SRA "runs" that failed to be identified in known datasets are outlined in a red square.

^{**:} All available SRA datasets from PRJNA607174

^{***:} Actual SRA datasets identified from the "Extended Data Table 3" of [2]

Analysis of reads from The Available datasets using NCBI Trace.

Table 2. The Trace result of Known GD Pangolin datasets when examined using NCBI Trace SRA.

	of Known GD Pangolin data	T	I
Accession number and	Primary Mammalian	Primate-related results	Identification of
registration date	Trace results and	in Krona and read size	"Coronaviridae"
	percentage	by Kbp	as by Trace and
			total read size
SRX6893158	Manis javanica: 14.66%	N/D	N/D
20-Sep-2019			
SRX6893157	Boreoeutheria: 1.24%	Catarrhini 644546	N/D***
20-Sep-2019			
SRX6893156	Manis javanica: 7.51 %	Homo sapiens 81948	Pangolin
20-Sep-2019	Homo sapiens: 0.03%		coronavirus 2Kbp
SRX6893155	Homo sapiens: 0.37%	Homininae 3534150	Pangolin
20-Sep-2019			coronavirus 5Kbp
SRX6893154	Homo sapiens: 0.02%	Hominoidea 356003	Pangolin
20-Sep-2019			coronavirus
			154Kbp
SRX6893153	Homo sapiens: 0.01%	Homo sapiens 162180	Pangolin
20-Sep-2019			coronavirus
			41Kbp
SRX6893152	Manis javanica: 2.87%	N/D	N/D
20-Sep-2019	Euarchontoglires: 1.37%		
SRX6893151	Manis javanica: 7.47%	N/D	N/D
20-Sep-2019			
SRX6893150	Boreoeutheria: 1.91%	N/D	N/D
20-Sep-2019			
SRX6893149	Manis javanica: 1%	Simiiformes 313069	N/D
20-Sep-2019			
SRX6893148	Manis javanica: 0.4%	Catarrhini 194320	N/D
20-Sep-2019			
SRX6893147	Manis javanica: 2.71%	Catarrhini 69937	N/D
20-Sep-2019			
SRX6893146	Boreoeutheria: 1.72%	Hominoidea 231755	N/D
20-Sep-2019			
SRX6893145	Homininae: 0.27%	Homininae 2536765	N/D
20-Sep-2019	Manis javanica: 1.01%		
SRX6893144	Manis javanica: 0.62 %	Hominoidea 166628	N/D
20-Sep-2019			
SRX6893143	Manis javanica: 1.63%	N/D	N/D
20-Sep-2019	-		
SRX6893142	Manis javanica: 1.28%	Simiiformes 57084	N/D
	1	l .	L

20-Sep-2019			
SRX6893141	Boreoeutheria: 1.41%	N/D	N/D
20-Sep-2019			
SRX6893140	Boreoeutheria: 1.56%	N/D	N/D
20-Sep-2019			
SRX6893139	Homo sapiens: 0.01%	Homo sapiens 491120	Pangolin
20-Sep-2019			coronavirus 2Kbp
SRX6893138	Boreoeutheria: 1.67%	Homininae 2761176	N/D
20-Sep-2019			
SRX7756769	Homo sapiens: 0.03%	Homo sapiens 5457929	Bat SARS-like
18-Feb-2020			coronavirus 2Kbp
			Wuhan seafood
			market
			pneumonia virus
			2Kbp
SRX7756766	Manis javanica: 78.6%	Cercopithecidae 3116	Betacoronavirus
18-Feb-2020			2Kbp**
SRX7756765	Manis javanica: 87.17%	Cercopithecinae 11339	N/D****
18-Feb-2020			
SRX7756764	Manis javanica: 48.39%	Cercopithecidae 22600	N/D
18-Feb-2020			
SRX7756763	Manis javanica: 94.95%	Cercopithecidae 5076	N/D
18-Feb-2020			
<u>SRX7756762</u>	Manis javanica: 95.37%	Catarrhini* 2831	Nidovirales 0Kbp
18-Feb-2020			
SRX7756761	Manis javanica: 13.63%	Chlorocebus sabaeus	N/D
18-Feb-2020		498506	
SRX7732094	N/A***	N/A	Pangolin
15-Feb-2020			coronavirus***

^{*:} Chlorocebus Sabaeus

Specific BLAST analysis

In order to determine the authenticity of the Primate-related reads in the datasets, Specific BLAST analysis is carried out for all datasets that possessed claimed or analyzed reads of coronaviridae-related viruses. An 100% full-length match that does not map to non-primates confirms Authenticity of read.

^{**:}Not claimed as being SARS-CoV-2 related in the original publication. Likely unrelated.

^{***}Not analyzable. All Non-Coronavirus data filtered out. Leaving only 2,633 reads, all of which can be mapped to the SARS-CoV-2 reference genome.

^{****8} reads as claimed by [10]



Fig.2a Specific BLAST analysis on the PRJNA607174 dataset, <u>SRX7756762</u>, that contained claimed SARS-CoV-2 related coronavirus reads. The 100% full-length matches clearly indicate presence of Primate-derived material.



Fig.2b BLAST result on the returned sequence revealed it as a Primate-derived MHC complex gene, confirming Primate origin.

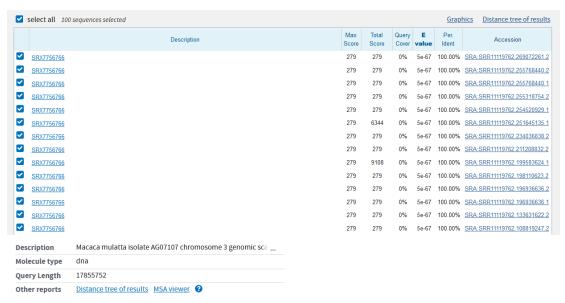


Fig.3a Specific BLAST analysis of <u>SRX7756766</u> revealed large amount of 100% full-length matches with Macaca Mulatta.



Fig.3b More intriguing—many of the reads showed only 100% matches to hominids—Chimpanzees and also clearly Macaca Mulatta itself. This indicate that SRX7756766 also contained significant amount of material derived from primates.



Fig.4a Similarly, <u>SRX7756769</u> contained large amount of reads that are 100% full-length matches to Human genomic DNA.



Fig.4b A BLAST analysis on reads sampled from the 100% hit results confirmed that it was found only in humans. Once again confirming human origin.

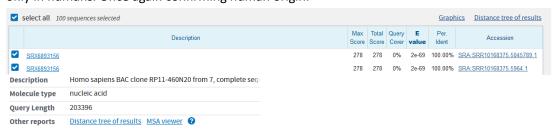


Fig.5a SRX6893156 also returned 100% matched results from the human Genome.



Fig.5b BLAST search on the result returned 100% match only found in humans. Confirming origin in human-derived material.

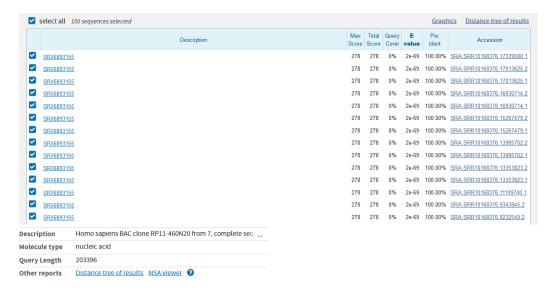


Fig.6a Similarly, BLAST research on <u>SRX6893155</u> gives large number of full length 100% matches to the human genome.

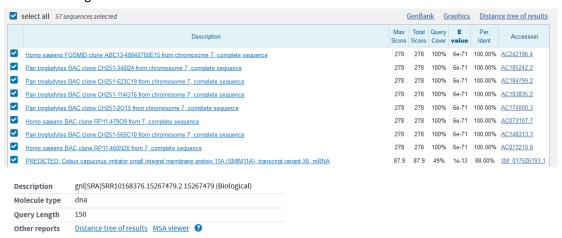


Fig.6b The results, when put through BLAST, confirms that the 100% matches are in fact derived from a Hominid origin.

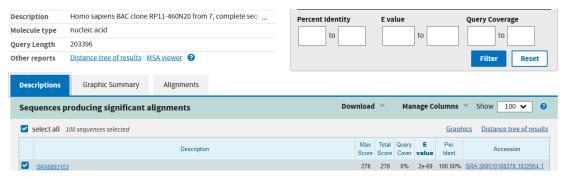


Fig.7a <u>SRX6893153</u> have also returned 100% match full-length read on this tiny part of the human genome.

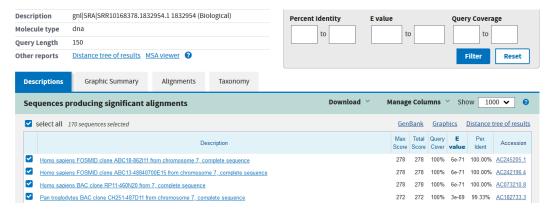


Fig.7b Similarly, the read is only found in humans—indicating the Homo Sapiens Trace result is accurate.

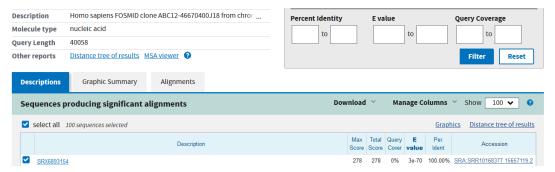


Fig.8a One read from the Human MHC gene is recovered from <u>SRX6893154</u> with a query sequence only 40058bp in length.

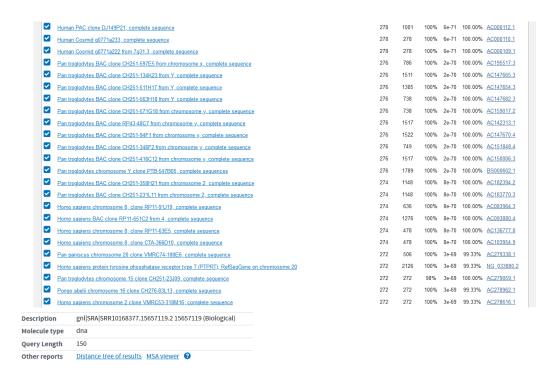


Fig.8b This MHC read is only found in Humans and Chimpanzees. This is clearly a contaminant from a hominid origin.

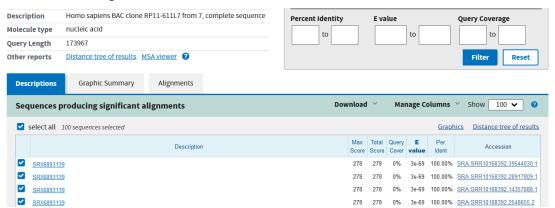


Fig.9a Similarly, multiple 100% match Full length reads were obtained from <u>SRX6893139</u>. As this query sequence is only 173967 nucleotides in length, the real extent of Human-derived contamination is also extremely severe.



Fig.9b Examining these reads revealed that they are only found in humans and apes. This is

therefore also clear evidence that there are Human/Hominid-derived contamination in SRX6893139.

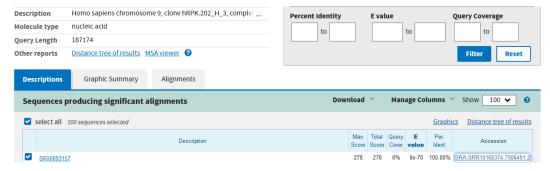


Fig.10a One read is also recovered from <u>SRX6893157</u>. From a query sequence only 187174nt in length.



Fig.10b This particular sequence is only found in humans—indicating that even the <u>SRX6893157</u> dataset was contaminated by material of human origin.

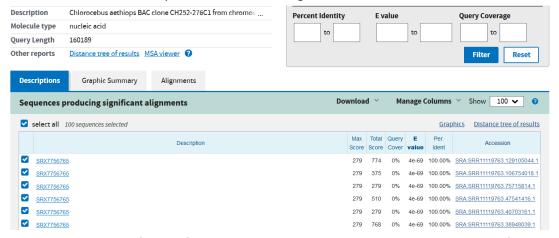


Fig.11a The presence of Reads from Somatic Chlorocebus aethiops in <u>SRX7756765</u> confirms the identity of the Cercopithecinae reads there.



Fig.11b the sequences from the BLAST hits indicate that they were unique to the family Cercopithecinae. Confirming Primate origin.

Analyzing the extent of contamination.

As the Specific BLAST analysis confirmed significant level of Human-derived contamination in all samples positive for SARS-CoV-2 related Coronaviruses, The TRACE result can therefore be trusted for the analysis on the extent of contamination.

The 32nt Krona Trace system is used for elucidating the ratio of different taxa within a sample. As Specific BLAST analysis confirmed the significant presence of Human and Primate derived Genetic material--The most basal group of primates detected in all Coronavirus-positive samples belong to Catarrhini—or Humans, Apes and Old-World Monkeys. Therefore, Trace classification results that can be classified into sister nodes of Catarrhini should be considered as Contamination by Primate-derived material.

Since Catarrhini is under Simiiformes; Haplorrhini; Primates; Euarchonta; Euarchontoglires and Manis is under Pholidota; Laurasiatheria, If a read is TRACEd down to Catarrhini, it can not be from a Pangolin, and it will have to be from a Primate-derived source—Contamination by material from the lab.

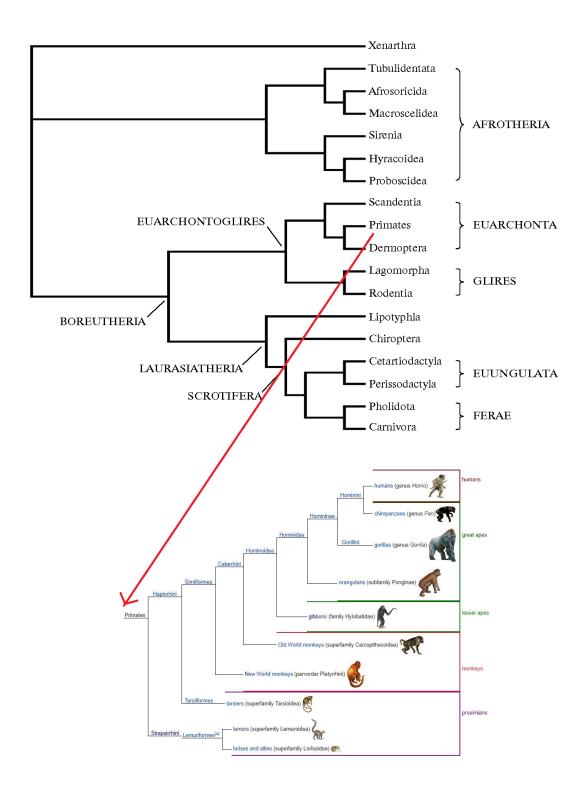


Fig. 12 Family tree of mammals, Including the position and classification of Primates in the lineage of Mammalia.

Table 3a Ratios of Hominid-traced reads to Pangolin-traced reads in the SRA datasets that contained reads of the GD- Pangolin-CoV sequence, and had Hominid reads.

Accession and	Primate	Total traced Kbps	Ratio of	Virus
date	classification and	to Manis Javanica	Primate to	classification
	total traced Kbps	(Pangolin)	Pangolin	and amount of
				reads by Kbps
SRX7756769	Homo sapiens	15401134	0.35	Bat SARS-like
18-Feb-2020	5457929			coronavirus
				2Kbp
				Wuhan seafood
				market
				pneumonia
				virus 2Kbp
SRX6893139	Homo sapiens	5301351	0.0926	Pangolin
20-Sep-2019	491120			coronavirus
				2Kbp
SRX6893157	Catarrhini	1889448	0.34	N/D***
20-Sep-2019	644546			
SRX6893156	Homo sapiens	4765461	0.01719	Pangolin
20-Sep-2019	81948			coronavirus
				2Kbp
SRX6893155	Homininae	525801	6.7214	Pangolin
20-Sep-2019	3534150			coronavirus
				5Kbp
SRX6893154	Hominoidea	2232008	0.159	Pangolin
20-Sep-2019	356003			coronavirus
				154Kbp
SRX6893153	Homo sapiens	3110158	0.05214	Pangolin
20-Sep-2019	162180			coronavirus
				41Kbp

^{***:} No trace result on Coronaviruses, despite claimed reads from [3]

Table 3b Ratios of Primate-traced reads to Coronavirus-traced reads in the SRA datasets that contained reads claimed to be traced to of the GD- Pangolin-CoV sequence, and lacked Hominid reads.

Accession and date	Primate classification	Virus	Ratio of virus
	and reads (in Kbp)	classification and	reads to
		reads	Primate reads
SRX7756766	Cercopithecidae 3116;	Betacoronavirus	0.000642
18-Feb-2020	BLAST to Macaca	2Kbp **	
	Mulatta		
SRX7756762	Catarrhini 2831;	Nidovirales 0Kbp	0.000530
18-Feb-2020	BLAST to Chlorocebus	Claimed	
	sabaeus	10x150bp reads	
SRX7756765	Cercopithecinae 11339	N/D***	N/A
22-Apr-2020	BLAST to Chlorocebus		
	Aethiops		
SRX7732094	N/A*	Pangolin	N/A*
15-Feb-2020		coronavirus	

^{*:} No non-coronavirus reads available in the dataset with a total of 2,633 reads, making analysis impossible.

DISCUSSIONS

The extent of contamination in the pangolin sequencing datasets

As the samples were supposed to be pangolin lung tissue, which will neither contact with nor be contaminated by non-pangolin derived mammalian tissues when still inside the animal, any non-pangolin mammalian reads within such a dataset can only be introduced to the sequencing process after the sample itself have been taken and brought into a lab.

As the classification Catarrhini itself is phylogenetically very deep down the Primate line which is itself distinguished from the Pangolin line at a very basal node (Boreoeutheria), and since we have already confirmed that the Primate line in PRJNA573298 traces mostly to humans by using Specific BLAST analysis, (SRX6893157, the only one of the claimed coronavirus read dataset that gives a classification just down to Catarrhini, contained 213 full length 100% matches to the Human Mitochondrial reference genome alone, which is only 16569 bp in length. All other datasets gives definitive TRACE mapping to Homo Sapiens and contained distinct 100% matched reads to even very small parts of the Human genome.), We can deduce the extent of contamination of the PRJNA573298 dataset by Primate-related materials as from a minimum of 1.6% to as high as 87% by sample mass—using the ratio of Primate reads to Pangolin reads on TRACE. Such high level of contamination with Primate-derived material is unacceptable for a sample that was supposed to be Lung tissue. And therefore, the virome data of such samples in PRJNA573298 no longer reflects the original virome of the animal, and an potential "novel" reads from these contaminated samples may have been from in-lab contamination instead.

^{**:} No claimed reads from [2]

^{***:} Claimed 8 reads from [10]

Deducing the dynamic of contamination in PRJNA607174

Of all 7 PRJNA607174 datasets, only <u>SRX7756769</u> and <u>SRX7756762</u> is claimed by Xiao et. Al to contain SARS-CoV-2-like reads. However, TRACE results revealed low level of contamination by Cercopithecidae (Old World Monkey) reads across all the samples. In particular, the <u>SRX7756762</u> dataset contained definitive mappings to Chlorocebus sabaeus, or African Green Monkey, while <u>SRX7756766</u> which contained 2Kbp unclaimed reads of Betacoronaviruses on TRACE, contained 100% full-length definitive mappings to Macaca Mulatta that may also be mapped to Homo Sapiens.

<u>SRX7756769</u> genetically resembles other samples in PRJNA573298, in both the kind of contamination and the extent of contamination. It contained an large excess of homo sapiens reads in levels similar to the contaminated samples in PRJNA573298.

From the method section of Lam et.al, we knew that they have performed Virus isolation using VERO E6 cells—Species Chlorocebus Sabaeus on one of the samples that have a positive PCR test for coronaviruses. The low level of contamination by Cercopithecidae-related reads in all the samples in PRJNA607174 except for SRX7756769 itself support the possibility that SRX7756769 is the first sample to be sequenced, and it happens before the lab begun using VERO E6 cells in the experiment. They then isolated the virus from the contaminated SRX7756769 in VERO E6 cells, characterized it but did not sequence it, and this cell culture material then contaminated SRX7756762 and possibly SRX7756766, resulting the 10 reads in SRX7756762 and the 2Kb batacoronavirus reads in SRX7756766.

The exact nature of SRX7732094 needs to be further scrutinized.

The P2S dataset, SRX7732094, displays very unusual property when compared to other Datasets under the same BioProject. It is the only dataset with all Non-coronavirus reads being filtered out, and contained too little spots for it to be an ILLUMINA NextSeq 550 run. Furthermore, it was the only dataset that did not contain metadata with either an isolation source or a Library prep procedure, other than "This dataset contains coronavirus-like sequence reads, based on BLAST search."

Such a strange designation and the fact of the dataset being heavily filtered, Raises problems on whether such a dataset is an actual BioSample at all. If this sample is really as claimed by Lam et. Al, Why the dataset have to be put through such heavy filtering when the other sequencing runs was clearly not filtered as severely as this dataset? Why there was no BioSample metadata on either Biomaterial provider, Source Tissue or Collector when all other Sequencing runs clearly provided such metadata information?

Unless the complete, unfiltered sequencing reads are made available on **SRX7732094**, and the rest of **PRJNA696875**, this Dataset can not be considered to be a real, reliable sample, and it must be excluded as "evidence" of a SARS-CoV-2-like virus infecting

Table 4 Sequencing runs in PRJNA696875, Accession number, BioSample, Content and designation

	T		T .	1	
Accession	Size	Non-Coronavirus	Source	Virus	Design
number and		reads?	Tissue	Designation:	
date			Provider	GD or GX?	
			and		
			Collected		
			by		
SRX7732094	2,633	No	N/A	GD	This dataset
15-Feb-2020					contains
					coronavirus-like
					sequence
					reads, based on
					BLAST search.
SRX7732093	470,344	Yes	Intestine	GX	NEBNext Ultra
15-Feb-2020			Yanling Hu		II DNA Library
			Wuchun		Prep Kit, paired
			Cao		sequencing
					data has been
					integrated.
SRX7732092	340,661	Yes	Lung	GX	NEBNext Ultra
15-Feb-2020			Yanling Hu		II DNA Library
			Wuchun		Prep Kit, paired
			Cao		sequencing
					data has been
					integrated.
SRX7732091	416,659	Yes	Intestine	GX	NEBNext Ultra
15-Feb-2020			Yanling Hu		II DNA Library
			Wuchun		Prep Kit, paired
			Cao		sequencing
					data has been
					integrated.
SRX7732090	520,254	Yes	Lung	GX	NEBNext Ultra
15-Feb-2020			Yanling Hu		II DNA Library
			Wuchun		Prep Kit, paired
			Cao		sequencing
					data has been
	L	1	L	l	

					integrate	d.
SRX7732089	19,607,536	Yes	Blood	GX	lon	Total
15-Feb-2020			Yanling Hu		RNA-Seq	Kit v2
			Wuchun			
			Cao			
SRX7732088	4,550,437	Yes	lung and	GX	lon	Total
15-Feb-2020			intestine		RNA-Seq	Kit v2
			Yanling Hu			
			Wuchun			
			Cao			

By closely examining the P2V dataset, SRX7732088, which claimed to be a culture sample in VERO E6 cells, Chlorocebus Sabaeus, the exact viral load in-culture when compared to Cellular mRNA can be deduced by dividing the total identifiable coronavirus signal to the total identifiable Primate signal within the dataset, 6943Kbp/451932Kbp, which correspond to 0.01536:1 Viral RNA to Cellular RNA.

This places the viral loads on the other datasets with Coronavirus-like reads from GD well within the threshold expected from cell culture contamination of the sequencing samples—including the samples in PRJNA607174.

Potential breach of data availability statement by Xiao et al.[2]

Sequence data that support the findings of this study have been deposited in GISAID with the accession numbers EPI_ISL_410721. Raw data of RNAseq are available from the NCBI SRA under the study accession number PRINA607174.

Fig 13. The Data Availability Statement of Xiao et al.

In the Data availability statement, the "Raw data of RNAseq" are clearly stated to be deposited under PRJNA607174. However, only 2 of the "Extended Data Table S3" datasets actually matches the datasets deposited on PRJNA607174. The other 7 datasets were completely unavailable. And the actual deposited datasets on PRJNA607174 does not match what have been claimed by Extended Data Table S3. As the RNA-seq Raw data was stated to be available within PRJNA607174, the failure to publish all the claimed data constitute a breach of the Data Availability statement on the article. Unless such datasets are published and independently examined, All such claimed reads from the strangely unpublished datasets can not be trusted as evidence of a SARS-CoV-2-like virus infecting pangolins in GuangDong, 2019.

Identifying the Etiological agent of the GuangDong 2019 incident.

By using an approach of both SRA TRACE analysis and specific BLAST Analysis, We have uncovered the fact that all samples that does not Contain confirmed Human-derived material, also lacked Claimed reads of a SARS-CoV-2 like virus that can be confirmed using NCBI Trace. All samples with claimed or traced reads of Coronaviruses in general, contained confirmed primate reads with the lowest common phylogenetic node Catarrhini. Samples that does not give a TRACE result on primate-derived material all lacked identifiable or claimed coronavirus reads.

This strongly imply that the Coronavirus-like reads are associated with human/Primate-sourced contamination material.

Most importantly, of all dead pangolins being sampled in the studies, only 9 out of a total of 29 Analyzable samples/datasets contained TRACEd or Claimed Coronavirus reads—despite all dead pangolins displayed similar symptoms in captivity. This imply that the alleged pangolin coronavirus is not the Etiological agent of the death of the pangolins being sampled in the studies. This is further supported by the fact that 4 out of 10 lung samples in PRJNA573298 and 4 out of 7 lung samples in PRJNA607174 lacked any claimed or TRACEd coronavirus reads—despite the same symptoms displayed and similar date of death.

In order to establish the Etiological agent of the dead pangolins in the single GuangDone Accident that leads to the sampling and studies. A full virome TRACE analysis is conducted on the available samples for the determining of the exact etiological agent.

Extended Data Table S1
Full virome TRACE results of all Analyzable datasets of the GD pangolin incident

	W	N-:: J	Murine respirovirus	P1::	N: J:1	Rubulavirus	N	Peribunyayi	A	C:_L	Siphoviridae	D-1
SRX6893158	Yes		No	No	No	No	Yes	No	Yes	Yes	No	No
SRX6893157	Yes	Yes	No	No	Claimed	No	No	Yes	No	No	No	No
SRX6893156	No	No	Yes	Yes	Yes	No	No	No	Yes	No	No	Yes
SRX6893155	No	No	Yes	No	Yes	No	No	No	No	No	No	No
SRX6893154	No	No	Yes	No	Yes	No	No	No	No	No	No	No
SRX6893153	No	No	Yes	Yes	Yes	No	No	No	Yes	No	No	No
SRX6893152	Yes	Yes	Yes	Yes	No	No	No	Yes	No	No	No	No
SRX6893151	Yes	Yes	No	Yes	No	No	No	Yes	Yes	No	No	No
SRX6893150	Yes	Yes	Yes	No	No	No	No	Yes	Yes	No	No	No
SRX6893149	Yes	Yes	No	No	No	No	No	No	No	No	Yes	No
SRX6893148	Yes	Yes	Yes	No	No	No	No	No	Yes	No	No	No
SRX6893147	Yes	Yes	"Respirovirus"	Yes	No	No	Yes	No	Yes	No	No	No
SRX6893146	Yes	Yes	Yes	No	No	No	No	No	No	No	No	No
SRX6893145	Yes	Yes	No	No	No	No	No	No	No	No	No	No
SRX6893144	Yes	Yes	Yes	Yes		No	No	No	No	No	No	No
SRX6893143	Yes	Yes	No	No	No	No	No	No	No	No	No	No
SRX6893142	Yes		No	No	No	No	No	Yes	Yes	No	No	No
SRX6893141	Yes	Yes	No	Yes	No	No	No	No	No	No	No	No
SRX6893140	Yes	Yes	Yes	No	No	No	No	Yes	No	No	No	No
SRX6893139	No	No	Yes	No	Yes	No	No	No	No	No	No	No
SRX6893138	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Yes	No	No	No
SRX7756766	No	No	Yes	Yes		Yes	No	No	No	No	No	No
SRX7756765	No	No	Yes	No	No	Yes	No	No	No	No	No	No
SRX7756764	No	No	Yes	No		Yes	No	No	No	No	No	No
SRX7756763	No	No	Yes	No	No	Yes	No	No	No	No	No	No
SRX7756762	No	No	Yes	No		Yes	No	No	No	No	No	No
SRX7756761 SRX7756769	No No	No No	Yes	No Yes	No Yes	Yes No	No No	No No	No No	No No	No No	No No
SKY1190109	INO	INO	Yes	ies	ies	INO	INO	INO	INO	INO	INO	INO

A full Virome TRACE result suggest all the dead pangolins were infected by either Mammarenaviruses or Murine Respirovirus, or both. Including both samples that contained Claimed Or TRACEd Coronavirus reads and the samples that didn't.

Murine Respirovirus and Mammarenaviruses co-infect 7 out of 29 Available Analyzable datasets, while None of the 29 datasets lacked both—indicating that both viruses were prevalent in the location where the pangolins were captive at The Guangdong Wildlife Rescue Center.

Symptoms of Murine Respirovirus in animals resembles that of SARS-CoV-2 in humans—It forms massive Syncytiums in Eukaryotic cells, suppresses the immune system and causes secondary bacterial infections. The virus causes necrosis of Lung tissue in 5 days, with similar inflammation and immunopathological effects in the lung tissues of infected animals [5]—creating the histopathological effect as reported by Xiao et al.

It should be worth pointing out that the only examined lung tissues were examined by Xiao et al. And all Lung tissue samples examined by Xiao et.al contained Reads from the Murine Respirovirus.

Similarly, Mammarenaviruses are also known to cause multi organ, lethal[7] infections, characterized by endothelial pathology and swelling of internal organs. [6] All of which were Symptoms reported in the incident. As these samples were not examined Histopathologically by

either the authors of [4] nor by any of the authors of any other article who have used the datasets/samples, leaving the only mean of elucidating the cause of death being the observed symptoms and the coarse examination of the organs during sampling. Mammarenavirus infection therefore remains the most likely cause of death of the Murine Respirovirus Negative samples in the available datasets.

Is the "GD pangolin CoV" really a virus of the pangolin?

The only examination of the binding affinity of the GD pangolin CoV RBD to different animal receptors was done by Xiao et al [2], which performed molecular dynamic simulation of the RBD docking to the Human ACE2 receptor, The Civet ACE2 receptor and the pangolin ACE2 receptor. If the RBD of GD pangolin CoV in deed evolved in pangolins, we should expect the binding affinity of the RBD toward the pangolin ACE2 receptor to be the highest binding affinity returned from the examination.

However, neither the GD pangolin CoV RBD, nor the RBD of SARS-CoV-2 which is highly similar, produced a higher binding affinity to the pangolin ACE2 receptor than to the human ACE2 receptor, and both binds the Human ACE2 receptor with the highest affinity across all 3 animal species (Human, Civet, Pangolin) examined.

This fact argues strongly against the RBD residues of the GD pangolin CoV being evolved in pangolins, and instead favoring the RBD and the virus being the result of a passage experiment of a possible virus of pangolin origin (The GX/P2V virus was isolated and passaged in VERO E6 cells during it's collection in 2017) in Primate-derived cell lines.

There are only 2 locations of Biological sample storage in GuangDong, the Guangdong Institute of Applied Biological Resources and the China National GeneBank.

As all Credible (Non-filtered and contained analyzable Non-Coronavirus reads) samples were collected in a single incident from the GuangDong Wildlife Rescue Center[1][4][2], which the initial sample collection and storage was carried out by the Guangdong Institute of Applied Biological Resources[4], this experimental culture likely contaminated the GD pangolin samples during their initial collection or Storage, Either by the lab worker doing the initial sampling, or during their storage in the facility.

Epidemiology analysis of SARS-CoV-2 and related viruses argues strongly against the existence of a Coronavirus with the claimed RBD residues and sequence similarity in or near the GuangDong Wildlife Rescue Center at the time and date of the incident and the collection of the samples.

The earliest collection date of the GD pangolin CoV available, MP789, GenBank MT084071.1, is displayed at 29 March 2019.

Since the original location of the animals and samples in question was inside the GuangDong Wildlife Rescue Center which is neither a certified Biosafety Laboratory nor possessed adequate

PPE when handling the animals, from the Simulation results by Xiao et al[2] and the observed high human transmissibility of SARS-CoV-2 which had a very similar RBD, Should the GD pangolin CoV genuinely exists at that date and within the unprotected GuangDong Wildlife Rescue Center, It would almost certainly infect one to multiple On-site workers (Rescue workers which lacked either the Biosafety training or the adequate PPEs required to handle tissues or animals infected with a virus as characterized by the GD pangolin CoV papers) in the GuangDong Wildlife Rescue Center, and caused a SARS-level epidemic in GuangDong 2013 beginning in or around April 2019. However, no such epidemic was recorded, nor there have been any virus that genetically resembled the GD pangolin CoV sequence (which is only 90% similar to SARS-CoV-2) being isolated in humans anywhere in the world even till today.

Nor there is a possibility that the current SARS-CoV-2 pandemic may have stemmed from the 29 March incident with the GD pangolin CoV, since the estimated time of divergence between the current SARS-CoV-2 genome to the GD pangolin CoV Genome was estimated to be at least 100 years ago , ranging from 1851 [1730,1958] to 1877 [1746,1986] [8], for a genome that is only 90% similar to SARS-CoV-2 and possessed significant difference in the sequence and composition of the viral proteins they encodes.

As the Earliest time of discovery and the incident on the GD pangolin CoV is no earlier than the beginning of Year 2019, The time between the incident and the first isolate of SARS-CoV-2 is far too short for GD pangolin CoV incident to be involved in the formation of the current SARS-CoV-2 pandemic, since even the neutral sites on the RBD itself would have taken more than 19.8 years to drift/evolve into what we seen today on the actual SARS-CoV-2 genome. [9]

Conclusions

The Extreme lack of transparency and the sheer level of contamination from the original samples, the lack of epidemiological evidence of it's existence at the location of it's collection, and the receptor binding affinity of the Viral RBD itself indicating it as not being evolved nor adapted in pangolins, all strongly argue against the existence of a SARS-CoV-2 like virus infecting pangolins captive in GuangDong at 2019.

Moreover, it suggests that the GD pangolin CoV exists only as a culture in Primate-derived cells within the lab/facility used for the initial collection and/or storage of the samples of the pangolins in question, raising important issues on the serial passage Gain-Of-Function research of viral pathogens.

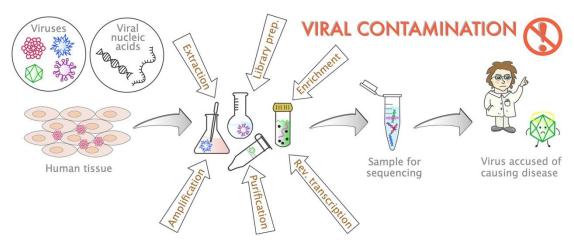


Figure 14. A cartoon diagram of contamination in sequencing experiment leading to false results and false "discoveries".

Note as in 2020/7/23

A recent Dataset, seemingly unrelated to the Xiao et.al Nature dataset, <u>SRX8582289</u>, appeared under <u>PRJNA607174</u>. This dataset seems to be newly sequenced, and it was not referred in [2].

Accession number and	Primary Ma	mmalian	Primate-related results	Identification of	
registration date	Trace results	and	in Krona and read size	"Coronaviridae"	
	percentage		by Kbp	as by Trace and	
				total read size	
SRX8582289	Manis javanica: 4	13.52%	Catarrhini 98913	Pangolin	
22-Jun-2020				coronavirus 792	

Table S2: TRACE analysis result of the SRX8582289 dataset.

Nevertheless, in-depth analysis revealed significant amount of contamination from the Human genome, with ratio of Virus to cell=0.8%.

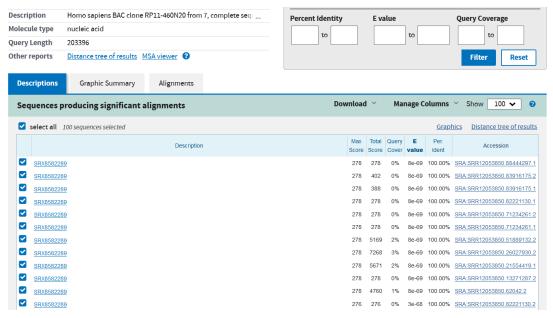


Figure S1A: Some BLAST hits out of a human Somatic BAC clone.

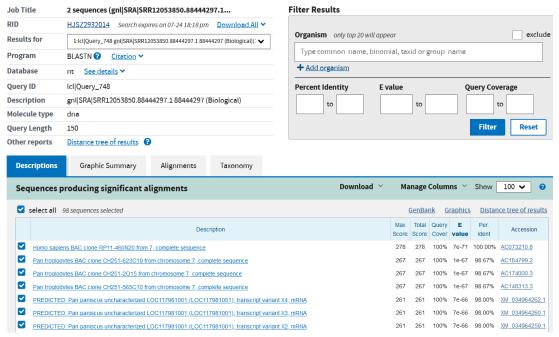


Fig. S1B: BLAST results returned only Homo Sapiens as 100% match. This indicate that the listed Catarrhini reads come from Homo Sapiens.

The significance of this particular dataset is yet unknown.

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