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

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ABSTRACT

Recently, there were much hype about reports of SARS-like coronaviruses being found in samples of Malayan pangolins (Manis Javanica) collected in Guangdong 2019, which appeared to possess nearly identical RBD's to the SARS-CoV-2 coronavirus. Prominent journals cited these discoveries to claim that pangolins may be 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 potentially contaminated. Here we also report that the presences of these 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 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 in the NCBI SRA. By Accession number, size and citation by thesis (if claimed to have SARS-CoV-2 related reads by paper).

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]		
SRX6893155	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			
SRX6893148	15,273,939			

SRX6893147	15,975,904	
SRX6893146	19,038,817	
SRX6893145	19,055,973	
SRX6893144	15,350,468	
SRX6893143	11,527,782	
SRX6893142	20,045,443	
SRX6893141	18,903,834	
SRX6893140	19,986,780	
SRX6893139	39,738,679	Lung02 [3] SRR10168392
SRX6893138	22,900,426	
<u>SRX7756769</u>	107,267,359 PRJNA607174**	M1[2]***
<u>SRX7756766</u>	273,651,431 PRJNA607174**	
SRX7756765	196,761,202 PRJNA607174**	
SRX7756764	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

_	ped	No. mapp	Total reads*	Animal species	Sample ID
RX7756769 "pangolin 9 "	←SR	496	107,267,359	Malayan pangolin	M1
		302	38,091,846	Malayan pangolin	M2
		14	79,477,358	Malayan pangolin	МЗ
lot available		1,100	32,829,850	Malayan pangolin	M4
		56	547,302,862	Malayan pangolin	M5
RX7756762 "pangolin 2"	←sı	10	232,433,120	Malayan pangolin	М6
	1	12	44,440,374	Malayan pangolin	М8
ot available	No	0	227,801,882	Malayan pangolin	M10
		0	444,573,526	Chinese pangolin	Z1

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.

Table 2. The Trace result of Known GD Pangolin datasets when examined using NCBI Trace SRA.						
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			
<u> </u>	1	1	ı			

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			
SRX7756762	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.

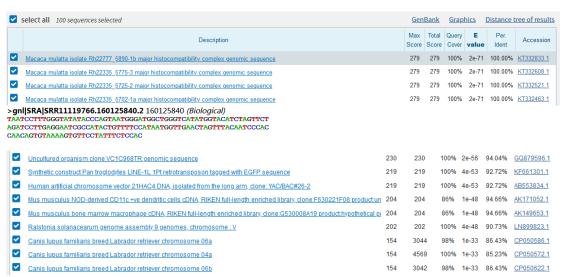


Fig.2b BLAST result on the returned sequence revealed it as a Primate-derived MHC complex gene that is not found in non-primates, confirming Primate origin.

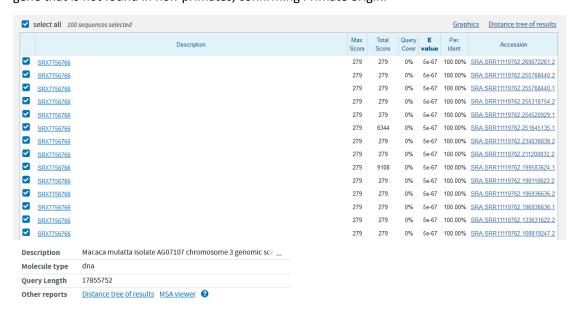


Fig.3a Specific BLAST analysis of <u>SRX7756766</u> revealed large amount of 100% full-length matches with Macaca Mulatta.

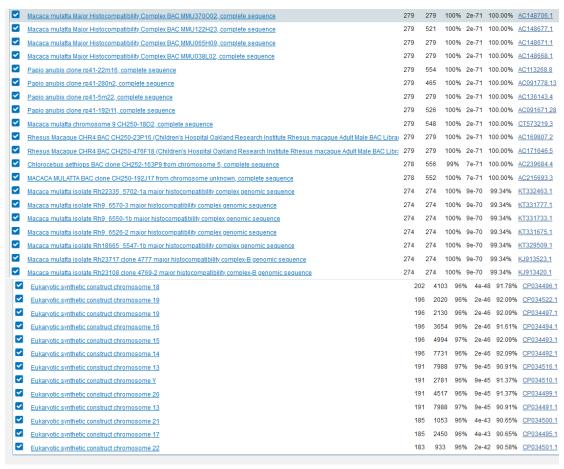


Fig. 3b BLASTing such matches gives 1005 matches to only Primates, and with no matches outside of Primates. This indicates that SRX7756766 also contained significant amount of material derived from primates.

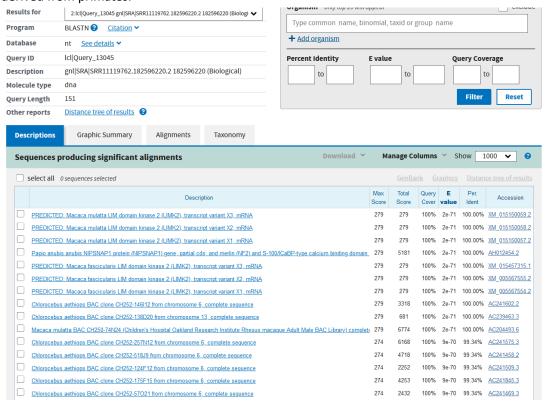


Fig.3C Presence of Primate-derived mRNA reads in <u>SRX7756766</u> confirms the Primate origin of these reads.



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

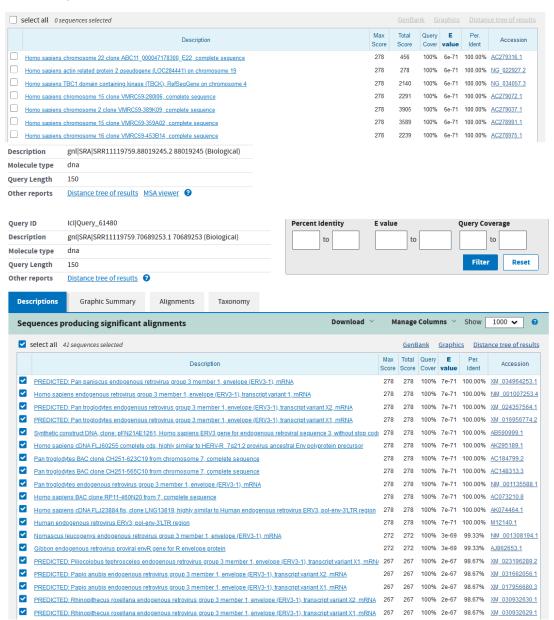


Fig.4b A BLAST analysis on reads sampled from the 100% hit results confirmed that it was found only in humans. Once again confirming human origin.

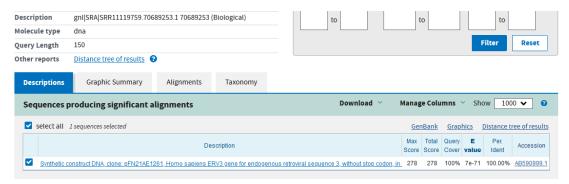


Fig.4c The sequence have no matches outside of Primates.

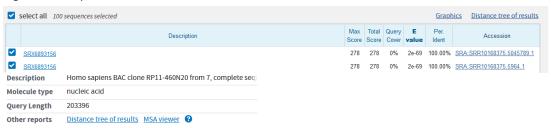


Fig.5a SRX6893156 also returned 100% matched results from the human Genome.

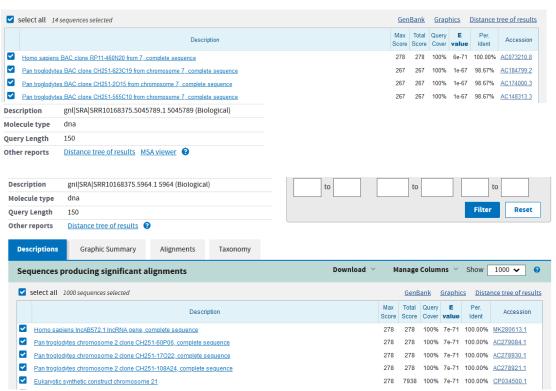


Fig.5b BLAST search on the result returned 100% match only found in humans, confirming origin in human-derived material.

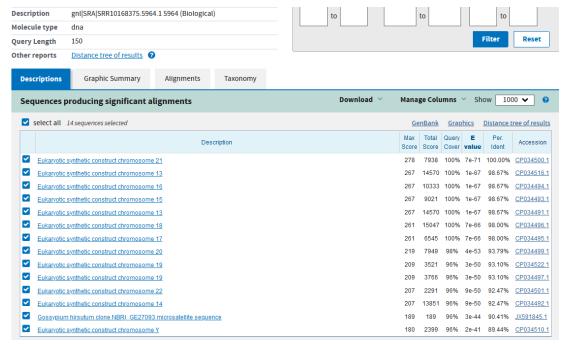


Fig.5c BLAST result of the sequences in question revealed that it is not found outside of Primates.

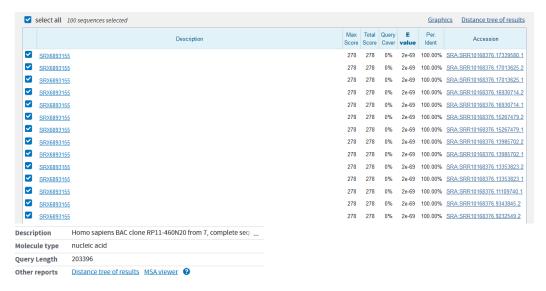


Fig.6a Similarly, BLAST research on <u>SRX6893155</u> gives large number of full length 100% matches to the human genome.

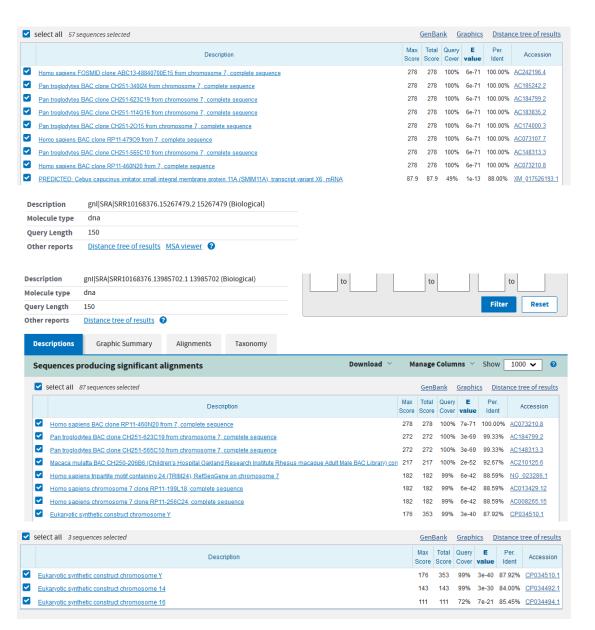


Fig.6b The results, when put through BLAST, confirms that the 100% matches are in fact derived from a Hominid origin.

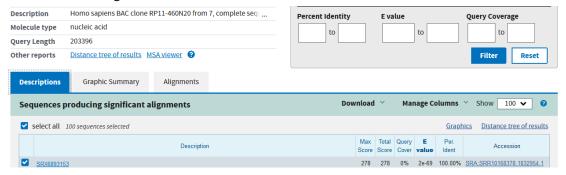


Fig.7a <u>SRX6893153</u> have also returned 100% match full-length read on this tiny part of the human genome.

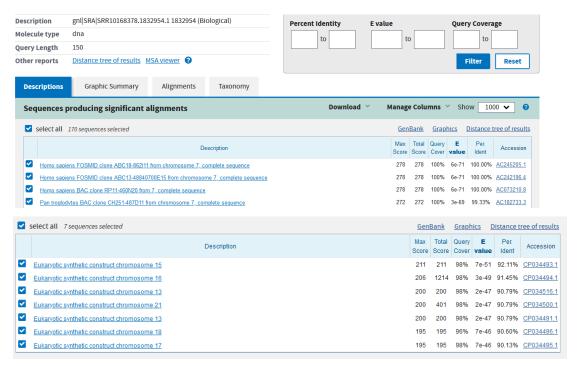


Fig.7b similarly, the read is only found in humans—indicating the Homo Sapiens Trace result is accurate.

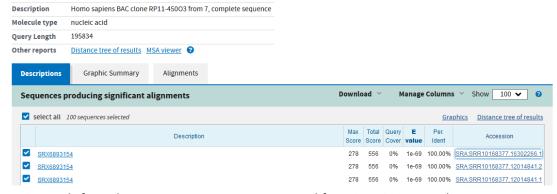


Fig.8a Reads from the Human PMS1 gene is recovered from <u>SRX6893154</u> with a query sequence only 195834bp in length.

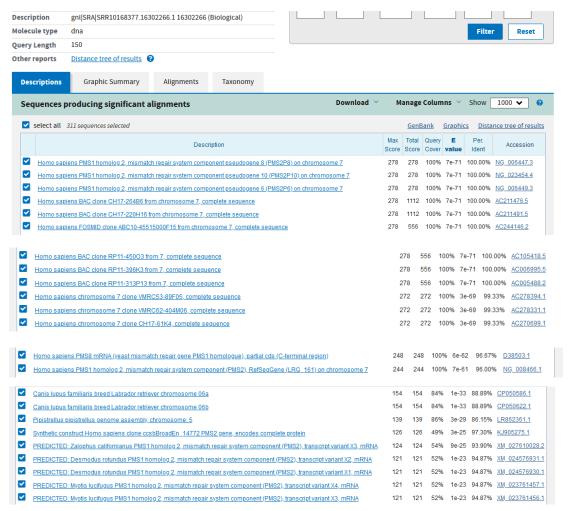


Fig.8b This PMS1 read is only found in Humans. This is clearly a contaminant from a hominid origin.

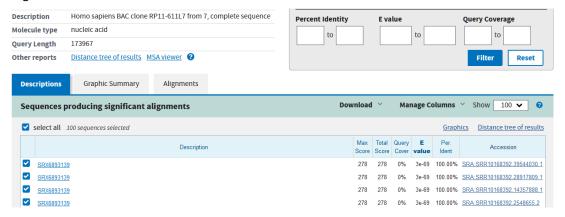


Fig.9a similarly, multiple 100% match Full length reads were obtained from <u>SRX6893139</u>. As this query sequence is only 173967 nucleotides in length, the real extent of Human-derived contamination is also extremely severe.

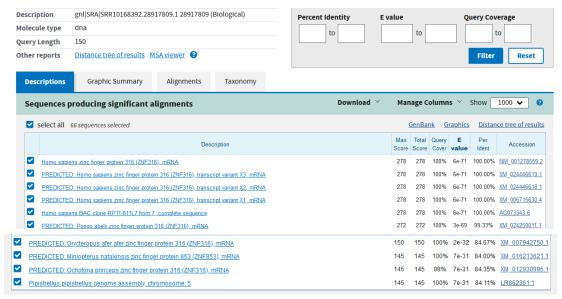


Fig.9b examining these reads revealed that they are only found in humans and apes. This is therefore also clear evidence that there is Human/Hominid-derived contamination in SRX6893139.

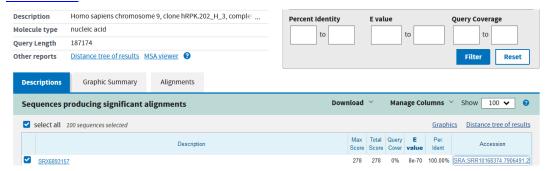


Fig.10a one read is also recovered from <u>SRX6893157</u>, from a query sequence only 187174nt in length.

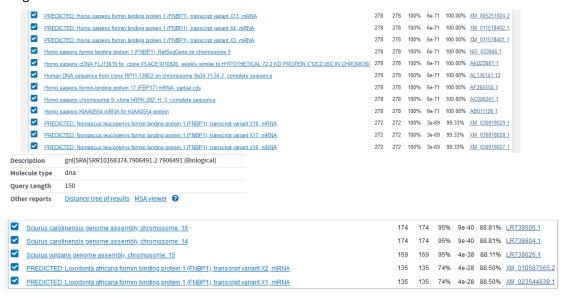


Fig.10b this particular sequence is only found in humans—indicating that even the <u>SRX6893157</u> dataset was contaminated by material of human origin.

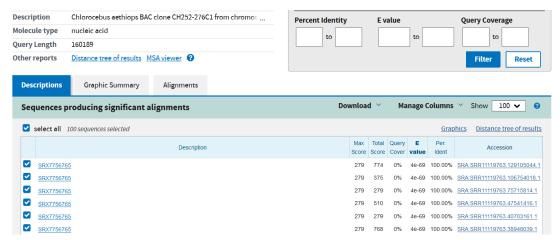


Fig.11a The presence of Reads from Somatic Chlorocebus aethiops in <u>SRX7756765</u> confirms the identity of the Cercopithecinae reads there.

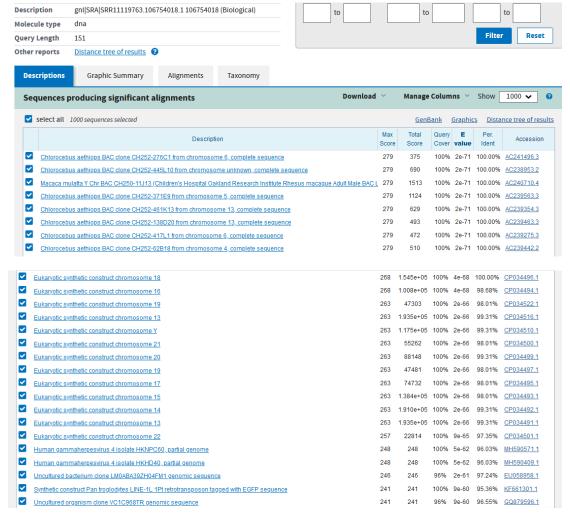


Fig.11b the sequences from the BLAST hits indicate that they were unique to the family Cercopithecinae. Confirming Primate origin.

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.

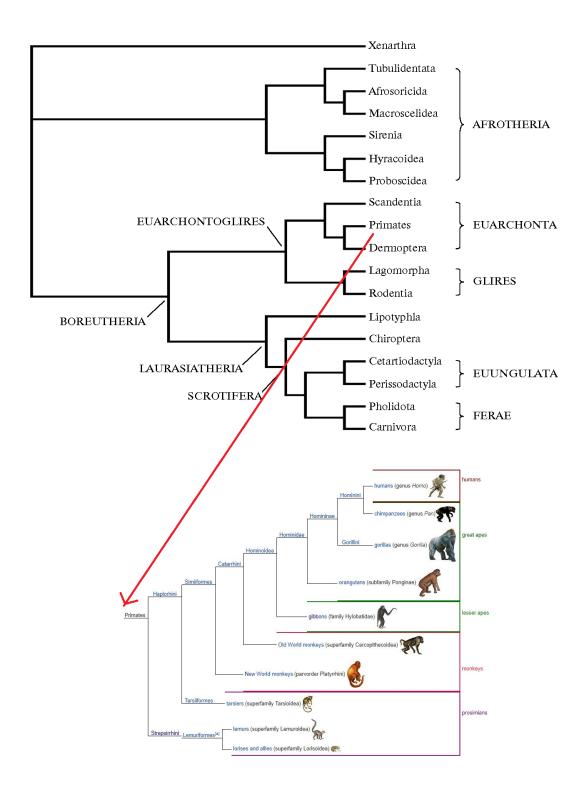


Fig. 12 Family tree of mammals, Including the position and classification of Primates in the lineage of Mammalia.

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 OKbp	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 Chlorocebus Aethiops and 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 **PRJNA606875**, 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

			_	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

					integrated	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

	Mammarenavirus	Nairoviridae	Murine respirovirus	Flaviviridae	Nidovirales	Rubulavirus	Nonanavirus	Peribunyayi	Amigovirus	Siphoviridae	Siphoviridae	Pahexavir
SRX6893158	Yes	Yes	No	No	No	No		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
SRX6893148	Yes	Yes	Yes	No	No		No	No	Yes	No	No	No
SRX6893147	Yes	Yes	"Respirovirus"	Yes	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
SRX6893144	Yes	Yes	Yes	Yes	No	No	No	No	No	No	No	No
SRX6893143	Yes	Yes	No	No	No	No		No	No	No	No	No
SRX6893142	Yes	Yes	No	No	No	No	No	Yes	Yes	No	No	No
SRX6893141	Yes	Yes	No	Yes	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
SRX6893138	Yes	Yes	Yes	Yes	No	No	Yes	Yes	Yes	No	No	No
SRX7756766		No	Yes	Yes				No	No	No		No
SRX7756765	No	No	Yes	No			No	No	No	No	No	No
SRX7756764	No	No	Yes	No			No	No	No	No	No	No
SRX7756763	No	No	Yes	No		Yes	No	No	No	No	No	No
SRX7756762	No	No	Yes	No		Yes	No	No	No	No	No	No
SRX7756761	No	No	Yes	No	No	Yes	No	No	No	No	No	No
SRX7756769	No	No	Yes	Yes	Yes	No	No	No	No	No	No	No

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 Syncytia 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]

Homo Sapiens reads are also found in SRX7756766, SRX7756765 and SRX7756762

In addition to Chlorocebus Spp. Indicative of VERO E6 cells, we have also found trace amount of reads uniquely matched to Hominidae within SRX7756766, SRX7756765 and SRX7756762. The presence of such reads may indicate that the Coronavirus-like reads from within such dataset were the result of index-hopping from a more highly contaminated original sample dataset, such as SRX7756769, of which the Homo Sapiens reads within such datasets may have index hopped into SRX7756766, SRX7756765 and SRX7756762, alongside with extremely low level of Coronavirus-related reads. Alternatively, these Homo Sapiens reads may represent the Homo Sapiens host sequence from the original sample which were left in the cell culture medium after inoculation of the VERO E6 cells as indicated by the virus isolation procedure performed by Xiao et al[2]. The presence of low levels of Homo Sapiens sequences within these datasets also confirms the origin of the inoculum into the VERO E6 cells as being samples that had significant amount of Homo Sapiens genetic material within them, which agrees with the hypothesis that the Coronavirus-related reads within these 3 datasets were the result of contamination by the In-lab VERO cell culture used by Xiao et al for virus isolation.

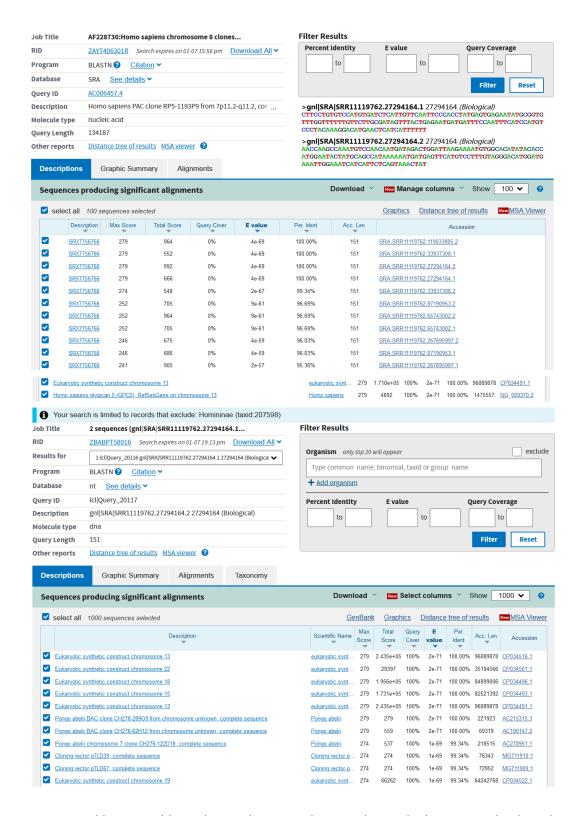


Fig 14: In addition to Chlorocebus Aethiops, reads uniquely matched to Hominidae have been found within SRX7756766.

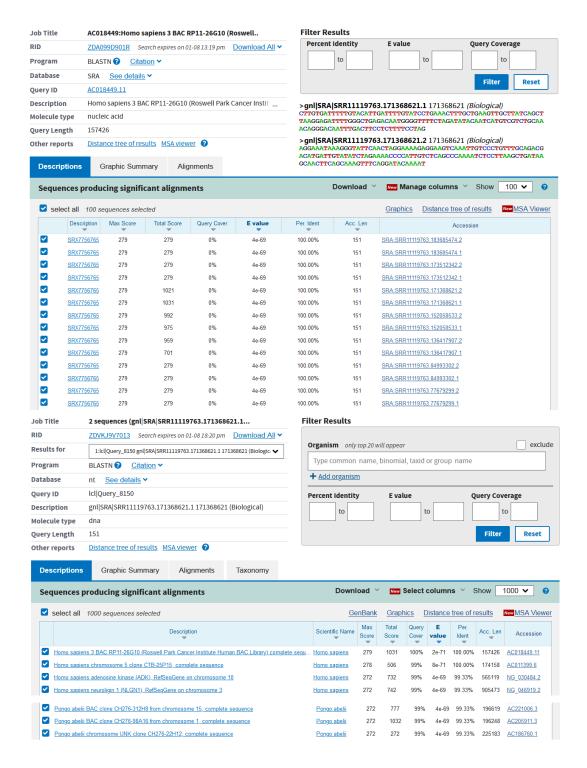


Fig 15: Reads uniquely matched to Homo Sapiens have been found within SRX7756765.

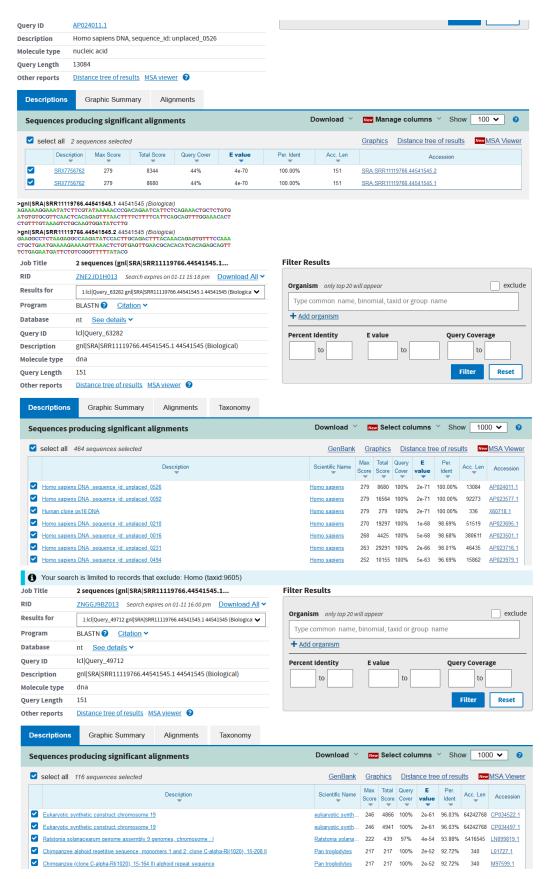


Fig. 16: Reads uniquely matched to Homo Sapiens Alpha Satellite DNA in SRX7756762.

The Pan-SL-CoV-GD sequences can not be found in other pangolin sequencing datasets from China.

Recently, we are able to access and perform BLAST analysis on a large dataset of 93 pangolin samples deposited by <u>Hu J et al.</u>,[12] located under the BioProject PRJNA529540. These samples, alongside with an older sample, SRX1319167, represent a longitudinal survey spanning from between 1990 to 2017 of Both Manis Pentadactyla and Manis Javanica from China. We could not obtain any traces of reads resembling the Pan-SL-CoV-GD sequences from these datasets. Such discovery is in agreement with the conclusion of <u>Hu J et al.</u>,[12] which failed to find any evidence of SARS-CoV-2-like Coronaviruses within their sequencing study.

Considering that another longitudinal survey of 334 pangolins in Malaysia[13] have also failed to reveal any evidence of Coronaviruses or other potentially zoonotic viruses, the failure to isolate sequences of Coronaviruses from pangolin sequencing datasets are in good agreement that no natural infection of a Coronavirus can happen to a pangolin in the wild. This may be due to their solitary behavior[14] which keep them completely physically isolated from each other for up to 9 months for each year when the population is not in it's mating season which happens from May to July. As this is longer than the time of which a pangolin could stay infected before either clearing the infection or dying in any known incidence of viral infection in captive pangolins[15], any virus species that enters a pangolin population in the wild will either be cleared or kills all of its current hosts when the population is not in or have left its mating season, resulting in the viral population to go extinct. Therefore, the absence of viral infections in pangolin populations is the normal state of such population, and any datasets that claimed viral infection of pangolins must be subjected to the highest level of scrutiny to exclude any potential presence of contamination.

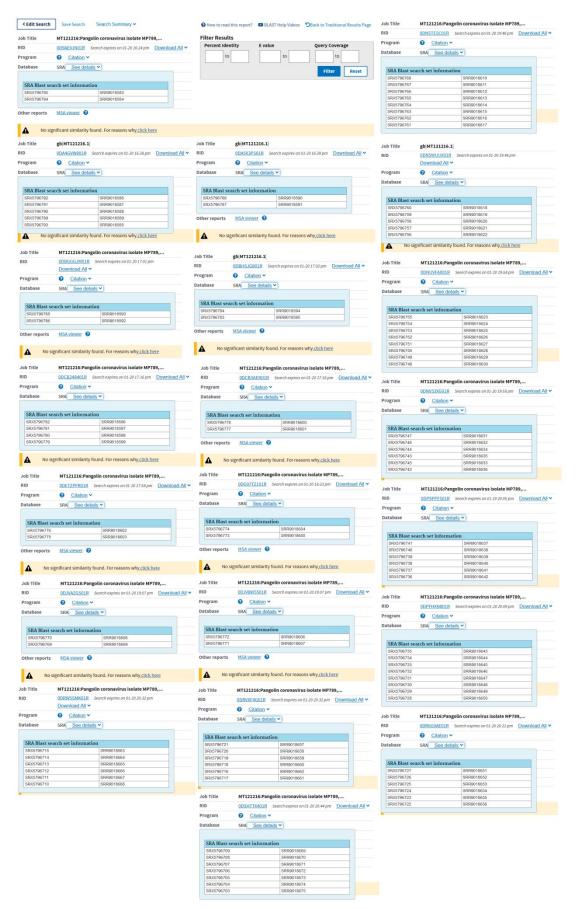


Fig.17a: No evidence of reads resembling the Pan-SL-CoV/GD sequences could be obtained from

PRJNA529540.



Fig17b: No evidence of reads resembling the Pan-SL-CoV/GD sequences could be found in SRX1319167.

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.

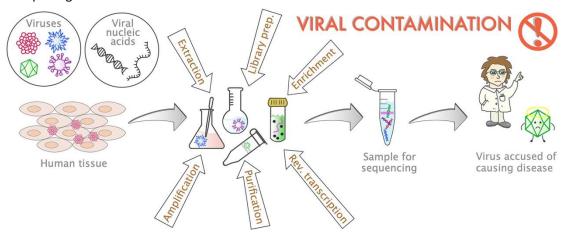


Figure 17. A cartoon diagram of contamination in sequencing experiment leading to false results and false "discoveries".

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].

Table S2: TRACE analysis result of the SRX8582289 dataset.

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
SRX8582289	Manis javanica: 43.52%	Catarrhini 98913	Pangolin
22-Jun-2020			coronavirus 792

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

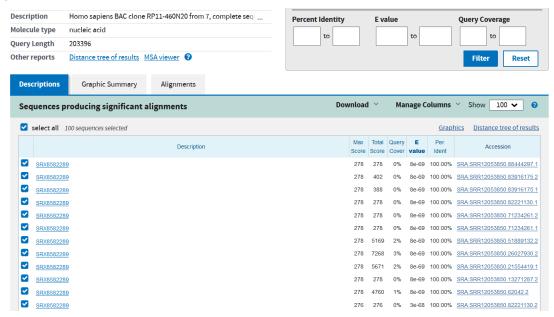


Figure S1A: Some BLAST hits out of a human Somatic BAC clone.

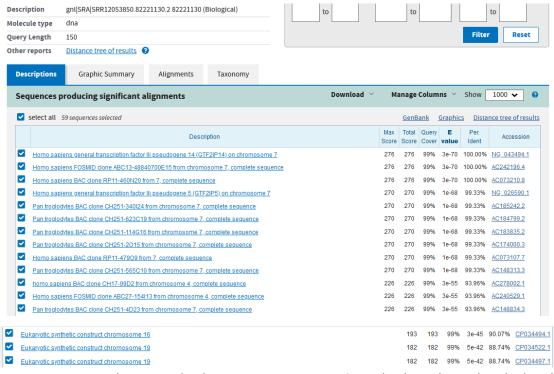


Fig. S1B: BLAST results returned only Homo Sapiens as 100% match. This indicate that the listed

Catarrhini reads come from Homo Sapiens.

The significance of this particular dataset is yet unknown.

Note as in 2020/12/26

Two Recent SRAs, <u>SRX9714436</u> and <u>SRX9714921</u>, were recently deposited by the Guangdong Institute of Applied Biological Resources with a listed DOI connection to 10.1371/journal.ppat.1008421 [1]. Both samples have a depositor of LinMao Li, 2020-12-21 the same time as the specified BioProject registration date. Only one of the SRAs contained significant amount of Coronavirus-related reads.

Table S3: TRACE analysis result of SRX9714436 and SRX9714921.

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
SRX9714436	Manis javanica: 3.14%	Homo sapiens 12332	Pangolin
	Homo sapiens: 0.04%		coronavirus 3
SRX9714921	Homo sapiens: 0.15%	Homo sapiens 9923	N/D

As expected by TRACE results, Reads that are 100% full-length uniquely matched to Homo Sapiens were obtained from SRX9714436 and SRX9714436

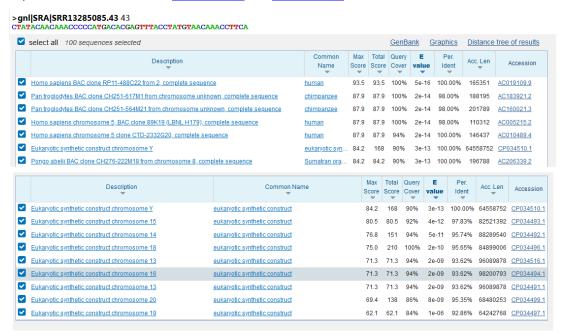


Fig.S2A: Homo Sapiens 100% full-length Unique matched read obtained from SRX9714436

>gnl SRA SRR13285570.36 36 ATAGGGAAGTGTGGTACCAAGGAGCAATATTCAATACAGCAACCAGGAAG									
	Description	Common Name	Max Score		Query	E value	Per. Ident	Acc. Len	Accession
	Human DNA sequence from clone XX-DSH1_29E11, complete sequence	human	93.5	93.5	100%	5e-16	100.00%	112322	CU041292.6
~	Human DNA sequence from clone RP11-535B18 on chromosome 9, complete sequence	human	93.5	93.5	100%	5e-16	100.00%	126815	AL354931.13

Fig.S2B: Homo Sapiens 100% full-length Unique matched read obtained from SRX9714921
A provided .fastq file was also found in SRX9714436. Analysis using stand-alone MagicBlast[11]

suggests significant presence of Homo Sapiens reads within this fastq file, similar to that of the Run itself.

87488

255

NDX550397 RUO:309:H3FKWBGXH:1:11101:21446:1055 16 AC019109.9

50M * 0 0 TGAAGGTTTGTTACATAGGTAAACTCGTGTCATGGGGGTTTGTTGTATAG * NH:i:1 AS:i:50 NM:i:0 Description Name Score Score Cover value Ident Acc. Len Accession ✓ Homo sapiens BAC clone RP11-488C22 from 2, complete sequence <u>human</u> 93.5 93.5 100% 5e-16 100.00% 165351 <u>AC019109.9</u> Pan troglodytes BAC clone CH251-617M1 from chromosome unknown, complete sequence chimpanzee 87.9 87.9 100% 2e-14 98.00% 188195 AC183921.2 Pan troglodytes BAC clone CH251-564M21 from chromosome unknown_complete_sequence chimpanzee 87.9 87.9 100% 2e-14 98.00% 201789 AC160021.3 Homo sapiens chromosome 5, BAC clone 89K19 (LBNL H179), complete sequence <u>human</u> <u>human</u> 87.9 87.9 100% 2e-14 98.00% 110312 AC005215.2 ✓ Homo sapiens chromosome 5 clone CTD-2332G20, complete sequence 87.9 87.9 94% 2e-14 100.00% 146437 AC010489.4
 Eukaryotic synthetic construct chromosome Y
 eukaryotic syn
 842
 168
 90%
 3e-13
 100,00%
 04050702
 97034910-1

 Pongo abelii BAC clone CH276-222M18 from chromosome 8, complete sequence
 Sumatran ora...
 842
 842
 90%
 3e-13
 100,00%
 196788
 AC206339.2
 <u>eukaryotic syn</u>... 84.2 168 90% 3e-13 100.00% 64558752 <u>CP034510.1</u>
 ✓ Human DNA sequence from clone RP11-987D21 on chromosome X, complete sequence
 human
 84.2
 84.2
 96%
 3e-13
 97.9%
 55442
 BX119919.5

 ✓ PREDICTED: Callithrix jacchus uncharacterized LOC118154814 (LOC118154814) n.GRNA
 white-futfled-e...
 82.4
 82.4
 94%
 1e-12
 97.87%
 2719
 XR_004745075.1
 ☑ Eukaryotic synthetic construct chromosome Y
   eukaryotic synthetic construct 84.2 168 90% 3e-13 100.00% 64558752 CP034510.1 eukaryotic synthetic construct ✓ Eukaryotic synthetic construct chromosome 15 80.5 80.5 92% 4e-12 97.83% 82521392 CP034493.1 Eukaryotic synthetic construct chromosome 14 76.8 151 94% 5e-11 95.74% 88289540 <u>CP034492.1</u> eukaryotic synthetic construct ☑ Eukaryotic synthetic construct chromosome 18 75.0 210 100% 2e-10 95.65% 84899006 <u>CP034496.1</u>

Fig.S3: BLAST result of the read NDX550397_RUO:309:H3FKWBGXH:1:11101:21446:1055 TGAAGGTTTGTTACATAGGTAAACTCGTGTCATGGGGGTTTGTTGTATAG within the provided fastq file. The read is 100% full-length uniquely matched to Homo Sapiens.

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