A sarbecovirus found in Russian bats uses human ACE2

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ABSTRACT

Spillover of sarbecoviruses from animals to humans resulted in outbreaks of severe acute respiratory syndrome SARS-CoVs and the ongoing COVID-19 pandemic. Efforts to identify the origins of SARS-CoV-1 and -2 has resulted in the discovery of numerous animal sarbecoviruses – the majority of which are only distantly related to known human pathogens and do not infect human cells. The receptor binding domain (RBD) on sarbecoviruses engages receptor molecules on the host cell and mediates cell invasion. Here, we tested the receptor tropism for RBDs from two sarbecoviruses found in Russian horseshoe bats to screen cell entry. While these two viruses are in a viral lineage distinct from SARS-CoV-1 and -2, one virus was capable of using human ACE2 to facilitate cell entry. Our findings demonstrate that sarbecoviruses circulating in wildlife outside of Asia also exhibit compatibility with human ACE2 and should be taken into consideration for future universal sarbecovirus vaccine candidates.

68 **Introduction**

69 Zoonotic spillover of sarbecoviruses from animals to humans has led to the
70 emergence of highly pathogenic human viruses, SARS-CoV-1 and -2, with the later
71 leading to the largest global pandemic in modern history. Researchers around the world
72 are ramping up the pace of viral discovery efforts, expanding the sequence databases with
73 new animal sarbecoviruses in circulation. While some laboratory experiments have been
74 performed with these new viruses, demonstrating a range of host tropisms, several viruses
75 remain untested, and thus their ability to transmit to humans is unknown.

76 Coronaviruses are covered with a "spike" glycoprotein that engages with receptor
77 molecules on the surface of host cells and mediates viral infection of the cell. A small
78 region within the spike proteins of sarbecoviruses, known as the receptor binding domain
79 (RBD), contains all of the structural information necessary to engage with the host
80 receptor. We and others have experimentally classified the majority of published
81 sarbecovirus RBDs into different clades based on sequence and functional data: clade 1,
82 identified in Asian bats, contains no deletions and binds to host receptor, Angiotensin-
83 Converting Enzyme 2 (ACE2), whereas clade 2, also identified in Asian bats, contains 2
84 deletions and does not use ACE2 and clade 3 viruses, found more widely in Africa and
85 Europe, contain 1 deletion and also infect using an unknown receptor[1-3]. Recently, several
86 viruses were identified in China that comprise a fourth clade that also interact with human
87 ACE2[4].

88 In late 2020, two clade 3 sarbecoviruses were identified in Rhinolophus bats in
89 Russia: Khosta-1 was found in *Rhinolophus ferrumequinum* and Khosta-2 in *R.*
90 *hipposideros*[5]. Similar to other European and African clade 3 viruses, the Khosta viruses

91 are divergent from the RBD found in SARS-CoV-1 and -2, and were thus presumed not to
92 possess any tropism for human cells. However, a recent study demonstrated that a new
93 clade (clade 4) of sarbecoviruses found in China containing a deletion similar to clade 3
94 sarbecoviruses can bind to human ACE2[4]. Here, we tested the RBDs from Russian
95 sarbecoviruses, along with other clade 3 viruses and report that the virus, Khosta-2, is
96 capable of infecting cells using the human orthologue of ACE2.
97
98
99 **Results**
100 Khosta virus receptor binding domains are distinct from human viruses
101 Khosta-1 and -2 were identified by Alkhovsky and colleagues in bat samples
102 collected between March-October 2020 near Sochi National Park[5]. Phylogenetic analysis
103 of the conserved viral gene, Orf1ab, revealed these viruses were most closely related to
104 another sarbecovirus found in Bulgaria in 2008 (known as BM48-31 or Bg08), and form a
105 lineage sarbecoviruses distinct from human pathogens, SARS-CoV-1 and -2[5]. A list of
106 viruses and accession numbers used in this study can be found in table 1. Phylogenetic
107 analysis of the spike RBD further reflected the close relatedness between Khosta -1 and -
108 2 with BM48-31 and other clade 3 RBD viruses we have previously tested from Uganda
109 and Rwanda[1,6] (Fig. 1a). Clade 3 RBDs, including the Khosta viruses, contain a truncated
110 surface-exposed loop, as compared to the ACE2-dependent, clade 1 viruses such as
111 SARS-CoV and additionally vary in most of the residues known for clade 1 viruses to
112 interact with ACE2[1,2,6,7].
113

114 RBD from Khosta viruses mediate entry into human cells

115 Using our scalable Sarbecovirus RBD entry platform, we replaced the RBD from
116 SARS-CoV-1 spike with the Khosta RBDs and generated chimeric spike expression
117 plasmids (Fig. 1b)[1]. For comparison, we also included chimeric RBD spikes for other clade
118 3 RBDs we have previously tested (BM48-31, Uganda, Rwanda) as well as SARS-CoV-2
119 and related RatG13 viruses. These chimeric spike expression constructs were used to
120 produce BSL2-compatible viral reporter pseudotypes with Vesicular Stomatitis Virus
121 expressing a dual GFP-luciferase reporter[1]. All of the chimeric spike proteins expressed
122 to similar levels in mammalian cells and incorporated in Vesicular Stomatitis Virus (VSV).
123 Chimeric spike with the RBD from BM48-31 and RatG13 showed reduced incorporation
124 but this did not correlate with viral entry phenotypes observed in later experiments (Fig.
125 1c, e).

126 To test human cell compatibility, we first infected the human liver cell line, Huh-7,
127 with pseudotypes bearing the chimeric Khosta RBD spikes. In the absence of the addition
128 of an exogenous protease, trypsin, the pseudotypes exhibited almost no entry in these
129 cells, which has been observed for other sarbecoviruses and is attributed to low
130 endogenous expression of ACE2. However, when trypsin was included during the
131 infection, entry signal strongly increased for SARS-CoV-1 and -2 RBDs as well as the
132 Khosta RBDs (Fig. 1d). As we and others have shown previously, trypsin enhancement of
133 sarbecovirus entry is still receptor dependent, suggesting that the Khosta virus RBDs were
134 using a receptor present in human cells to mediate infection[1,8].

135

136

137 The RBD from Khosta-2 infects cells using human ACE2

138 To characterize potential receptors that the Khosta viruses may be using, we
139 performed a classic receptor tropism test, where we transfected Baby Hamster Kidney
140 (BHK) cells with human orthologues of known coronavirus receptors and then infected with
141 our pseudotype panel. Unlike 293T cells, which express low levels of human ACE2 and
142 potentially other coronavirus receptors and have been shown to have low but measurable
143 permissivity to SARS-CoV infection, BHK cells are generally considered completely non-
144 permissive for sarbecoviruses unless a suitable receptor is supplemented[9]. The Khosta-1
145 RBD failed to infect cells expressing any of the receptors, while Khosta 2 RBD clearly
146 infected cells expressing human ACE2 (figure 1e). The level of cell entry mediated by the
147 Khosta 2 RBD was similar to RatG13, a bat sarbecovirus with high similarity to SARS-
148 CoV-2 in the RBD that also binds human ACE2, albeit with lower efficiency than the human
149 pathogen[10,11] (Fig. 1e). In contrast to the ACE2 results, only HCoV-229E could infect cells
150 expressing Aminopeptidase N (APN), and MERS-CoV spike could only infect cells
151 expressing dipeptidyl peptidase IV (DPP4) – the known receptors for these viruses,
152 demonstrating these receptors were expressed to functional levels (Fig. 1e).

153

154 **Discussion**

155 To date, all animal derived sarbecoviruses capable of infecting cells through
156 human ACE2 have been found in Asia[2,12-15]. Here, we provide evidence that animal-
157 derived sarbcoviruses circulating in wildlife outside of Asia can also possess the capacity
158 to infect human cells and use human ACE2 (Fig. 1). Khosta 1 and 2 viruses are most
159 closely related to other clade 3 RBD viruses, which have been found across a much wider

160 geographic range than the clade 1 viruses[1,6,16,17]. Thus, our findings show that
161 sarbecovirus host receptor compatibility is not geographically locked and that other
162 sarbecoviruses, which may only be distantly related to human SARS-CoVs, may also
163 stochastically possess the capacity to infect humans. Importantly, as the researchers who
164 initially discovered the Khosta viruses note with their findings: the Khosta bat
165 sarbecoviruses are genetically distinct from human SARS-CoVs in that they lack genetic
166 information encoding for some of the genes thought to antagonize the immune system and
167 contribute to pathogenicity, such as Orf8[5]. Unfortunately, because coronaviruses are
168 known to recombine in co-infected hosts, the recent identification of SARS-CoV-2 spillover
169 from humans back in wildlife populations opens the possibility of new human-compatible
170 sarbecoviruses[18-23].

171 In the presence of trypsin, both Khosta-1 and -2 RBDs were capable of infecting
172 human cells, with Khosta-1 performing notably stronger than Khosta-2, however in our
173 receptor-specific assay, only Khosta-2 could infect cells expressing human ACE2 without
174 exogenous protease (Fig. 1d, e). We have previously shown that a small number of clade
175 2 RBDs, such as As6526, also exhibit protease-mediated, ACE2-independent entry, and
176 similar phenotypes have been described for other bat coronaviruses[1,24]. Because not all
177 of the clade 2 and 3 viruses exhibit this phenotype, these findings collectively suggest that
178 other coronaviruses can infect human cells through a presently unknown receptor.
179 Sarbecoviruses have been shown to co-circulate in their reservoirs, so this variation in
180 receptor usage among closely related viruses may even represent an evolutionary strategy
181 for viral persistence[2].

182 Although the Khosta-2 RBD exhibited clear compatibility with human ACE2,
183 pseudotype entry was approximately 10-fold less than for SARS-CoV-1 and -2, and was
184 to levels closer to RatG13 RBD (Fig. 1e). The RBD for RatG13, while also compatible with
185 human ACE2, has been shown by other groups to exhibit poor binding to human ACE2[25-
186 27]. A single point mutation, T403R/K, has been shown to improve RatG13-RBD binding
187 and entry with human ACE2, although this mutation is already present in Khosta-2
188 (K403)[25]. We have previously demonstrated that MERS-CoV can rapidly adapt to
189 overcome a semi-permissive receptor, suggesting that limited entry phenotypes are easily
190 overcome by betacoronaviruses[28]. As new sarbecoviruses are discovered in wildlife, there
191 is a propensity in the research community to compare single amino acids based on known
192 human SARS-CoVs as an indicator for compatibility with humans. Our work underscores
193 the challenges in predicting viral function from sequence data alone and sheds light on
194 another group of sarbecoviruses with zoonotic potential. Current universal sarbecovirus
195 vaccines in development include mostly clade 1 viruses and one of the clade 2 viruses but
196 do not include any members from clade 3[29,30]. Taken together, our findings with the Khosta
197 viruses show that the sarbecoviruses may pose an even broader geographic threat than
198 previously appreciated.
199
200 **Methods**
201 Phylogenetic analysis
202 Genbank accession numbers for all sarbecovirus spike sequences used in this study are
203 available in table 1. Amino acid sequences for the receptor binding domain of the spike
204 glycoprotein were aligned using ClustalW multiple sequence alignment with default

205 parameters. A maximum likelihood phylogenetic tree was inferred with PhyML v. 3.0[31]
206 using the 'WAG' matrix +G model of amino acid substitution as selected by Smart Model
207 Selection method with 1000 bootstrap replicates[32]. The final tree was then visualized as a
208 cladogram with FigTree v1.4.4 (https://github.com/rambaut/figtree).
209
210 Plasmids and sequences
211 Untagged human orthologues of ACE2 (Q9BYF1.2), APN (NP_001141.2), and DPP4
212 (XM_005246371.3) were described previously[1]. Spike sequences from HCoV-229E
213 (AB691763.1), MERS-CoV (JX869059.2), and SARS-CoV-1 (AY278741) were codon-
214 optimized, appended with a carboxy-terminal FLAG tag sequence separated by a flexible
215 poly-glycine linker and cloned into pcDNA3.1+ as previously described[1]. The SARS-CoV-
216 1 RBD was removed with KpnI and XhoI and replaced with codon-optimized, synthesized
217 RBD fragments as previously described[1].
218
219 Cells and pseudotype assay
220 293T, Huh-7 (human liver cells), and BHKs were maintained under standard cell culture
221 conditions in DMEM with L-glutamine, antibiotics, and 10% Fetal Bovine Serum. Single-
222 cycle, Vesicular Stomatitis Virus (VSV) pseudotype assays were performed as previously
223 described[1]. Briefly, 293T "producer cells" were transfected with spike plasmids or empty
224 vector as a "no spike" control and infected 24-hours later with VSV-ΔG-luc/GFP particles,
225 generating chimeric-spike pseudotyped particles that were harvested 72 hours post-
226 transfection and stored at -80°C. Target cells were plated in 96-well format, and spin-
227 infected in quadruplicate with equivalent volumes of viral pseudotypes at 1200xG for 1

228 hour at 4°C. Infected cells were incubated for approximately 18-20 hours and luciferase
229 was measured using the Promega BrightGlo luciferase kit following manufacturers'
230 instructions (Promega). Entry signal was normalized to the average signal for the "no
231 spike" control. Plates were measured and analyzed in triplicate. Data are representative
232 of four complete biological replicates.
233
234 Westernblot
235 Viral pseudotypes were concentrated and 293T producer cells were lysed in 1% SDS and
236 clarified as described previously[1]. Lysates were analyzed on 10% Bis-Tris PAGE-gel
237 (Thermo Fisher Scientific) and probed for FLAG (Sigma-Aldrich; A8592; 1:10000); GAPDH
238 (Sigma-Aldrich, G8795, 1:10000); and/or VSV-M (Kerafast, 23H12, 1:5000). Signal was
239 detected using SuperSignal West substrate (Thermo-Fisher).
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249 **AUTHOR CONTRIBUTIONS STATEMENT**
250 S.N.S. and M.L. performed experiments and wrote the manuscript. M.L. designed the
251 study, analyzed the data, and assembled the figures.
252
253
254 **ADDITIONAL INFORMATION**
255 The authors declare no competing interests.

Figures, figure legends and tables

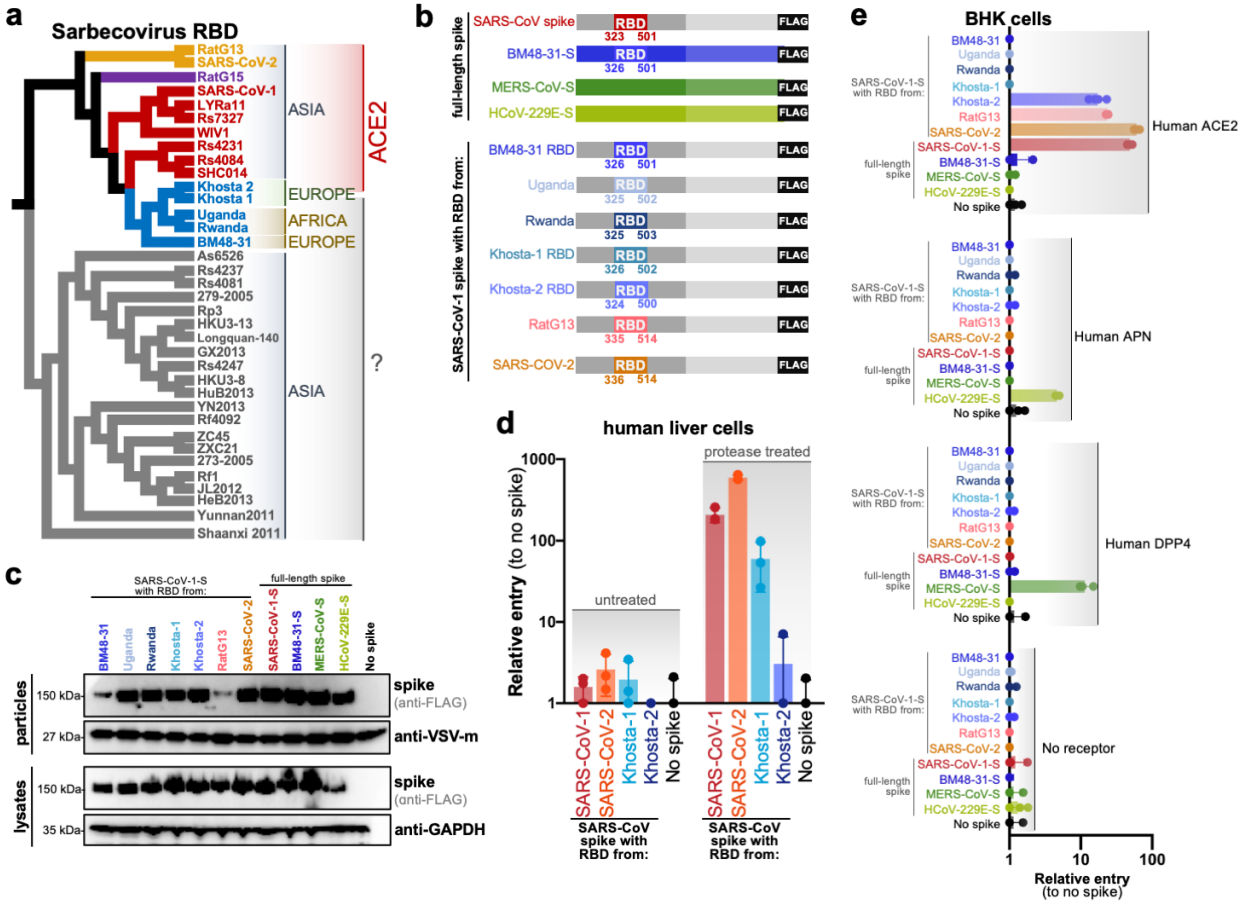

Figure 1. Khosta-2 uses human ACE2 to infect cells. (a) Sarbecovirus Receptor Binding Domain Cladogram based on amino acid sequenced. Countries of origin and known host receptors are indicated to the right. Clade 1 viruses are shown in red and orange, clade 2 in grey, clade 3 in blue and clade 4 in purple. (**b**) Diagram of spike constructs used for this study. The SARS-CoV-1 RBD was replaced with RBDs from other sarbecoviruses. (**c**) Expression and incorporation of viral pseudotypes by westernblot. (**d**) Huh-7 cells were infected with pseudotypes in the presence of absence of trypsin. Cells were infected in triplicate. (**e**) BHK cells were transfected with receptors and infected in the absence of trypsin. Cells were infected in quadruplicate.

Table 1. Sarbecovirus sequences used in this study

	Virus	Accession	Clade	Host species	Location
1	SARS Urbani	AY278741	1	human	Guangdong, China (origin of outbreak)
2	WIV1	KF367457	1	*Rhinolophus sinicus*	Yunnan, China
3	LYRa11	KF569996	1	*Rhinolophus affinis*	Baoshan, Yunnan, China
4	Rs7327	KY417151	1	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
5	Rs4231	KY417146	1	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
6	Rs4084	KY417144	1	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
7	RsSHC014	KC881005	1	*Rhinolophus sinicus*	Yunnan, China
8	SARS-CoV-2	MN908947	1	human	Wuhan, Hubei, China
9	RatG13	MN996532	1	*Rhinolophus affinis*	Yunnan, China
10	RatG15	*from preprint*	4	*Rhinolophus affinis*	Mojiang County, Yunnan Prvince, China
11	As6526	KY417142	2	*Aselliscus stoliczkanus*	Kunming, Yunnan Province, China
12	Yunnan2011	JX993988	2	*Chaerephon plicata*	Yunnan, China
13	Shaanxi2011	JX993987	2	*Rhinolophus pusillus*	Shaanxi, China
14	279-2005	DQ648857	2	*Rhinolophus macrotis*	Hubei, China
15	Rs4237	KY417147	2	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
16	Rs4081	KY417143	2	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
17	Rp3	DQ071615	2	*Rhinolophus pearsoni*	Nanning, Guangxi, China
18	Rs4247	KY417148	2	*Rhinolophus sinicus*	Kunming, Yunnan Province, China
19	HKU3-8	GQ153543	2	*Rhinolophus sinicus*	Guangdong, China
20	HKU3-13	GQ153548	2	*Rhinolophus sinicus*	Guangdong, China
21	GX2013	KJ473815	2	*Rhinolophus sinicus*	Guangxi, China
22	Longquan-140	KF294457	2	*Rhinolophus monoceros*	Longquan, Zhejiang, China
23	YN2013	KJ473816	2	*Rhinolophus sinicus*	Yunnan, China
24	Rf4092	KY417145	2	*Rhinolophus ferrumequinum*	Kunming, Yunnan Province, China
25	ZXC21	MG772934	2	*Rhinolophus sinicus*	Zhoushan City, Zhejiang, China
26	ZC45	MG772933	2	*Rhinolophus sinicus*	Zhoushan City, Zhejiang, China
27	JL2012	KJ473811	2	*Rhinolophus ferrumequinum*	Jilin, China
28	HuB2013	KJ473814	2	*Rhinolophus sinicus*	Hubei, China
29	Rf1	DQ412042	2	*Rhinolophus ferrumequinum*	Yichang, Hubei, China
30	HeB2013	KJ473812	2	*Rhinolophus ferrumequinum*	Hebei, China
31	273-2005	DQ648856	2	*Rhinolophus ferrumequinum*	Hubei, China
32	BM48-31	NC014470	3	*Rhinolophus blasii*	Strandja Nature Park, Bulgaira
33	Uganda	MT726044	3	*Rhinolophus ferrumequinum*	Uganda
34	Rwanda	MT726045	3	*Rhinolophus ferrumequinum*	Rwanda
35	Khosta 1	MZ190137	3	*Rhinolophus ferrumequinum*	Greater Caucasus , Russia
36	Khosta 2	MZ190138	3	*Rhinolophus hipposideros*	Greater Caucasus , Russia

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