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Candida vulturna Outbreak Caused by Cluster of Multidrug-Resistant Strains, China

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Appendix

Additional Case Details

Case 4 was initially admitted to the department of geriatrics; Case 6 the department of general surgery; Cases 7 and 10 the department of neurosurgery; and Case 8 the department of orthopedics. These patients were later transferred to the ICU of the hospital. The earliest two *C*. *vulturna* infection cases were identified in the neuroscience ward, we suspect that *C. vulturna* was transmitted from other wards to the ICU.

Some patients were initially admitted to the general surgery, neuroscience, or other wards of the hospital prior to transfer to the ICU. Since the earliest two *C. vulturna* infection cases were identified in the neuroscience ward, we suspect that *C. vulturna* was transmitted from other wards to the ICU.

There could be multiple reasons for the reduction of infection cases during COVID. First, during COVID, the disinfectant with an increased concentration of hypochlorite (two-fold) was

used for floor disinfection. Second, disinfectants (such as 75% alcohol in the form of sprays and wipes) were available for all visitors and healthcare staff throughout the hospital. Third, the general and ICU wards strictly limited visitors.

Materials and methods

Strains and culture conditions

C. vulturna, *C. auris*, and *C. haemuloni* strains were routinely grown on solid YPD medium (2% Glucose, 2% peptone, 1% yeast extract, 2% agar). Modified Lee's glucose media (1) was used for *Candida* aggregation and biofilm assays.

For growth on nutrient agar, approximately 150 cells were plated on Lee's glucose medium and cultured at 30°C or 37°C for 3 days. For liquid culture, fungal cells were inoculated into 3 mL Lee's glucose liquid medium to an OD₆₀₀ of 0.2 and incubated at 30°C or 37°C with shaking for 24 hours.

Environmental screening assays were performed to isolate *C. vulturna* from hospital surfaces, including walls, floors, bedside tables, bed sheets, bed rails, bed frames, blood-pressure cuffs, and chairs. More than 300 environmental samples were analyzed. Swab and wipe samples were used for culture assays on CHROMagar *Candida* medium.

To develop biofilms on silicone squares, approximately 2 x 10^6 cells of each strain were inoculated into each well containing one silicone square (10 mm x 10 mm, Bentec Medical, INC., Woodland) and 600 µL Lee's glucose medium. After incubation for 48 hours at 30°C with shaking, the silicone squares were washed gently with ddH₂0 three times and used for scanning electron microscopy (SEM) assays.

SEM assays

SEM assays were performed as described in our previous publication (2). Briefly, the silicone squares with *Candida* biofilms were fixed with 2.5% glutaraldehyde. The samples were

dehydrated in increasing concentrations of ethanol (25%-50%-70%-90%-100%), followed by tert-butyl alcohol solvent displacement through a series of increasing concentration of tert-butyl alcohol (25%-50%-70%-90%-100%) and freeze-dried. Finally, the samples were coated with gold and imaged using a scanning electron microscope (FlexSEM 1000 II, HITACHI).

Antifungal drug susceptibility assays

Minimum inhibitory concentrations (MICs) were determined according to the CLSI (Clinical Laboratory Standards Institute, 2012) guidelines. Liquid RPMI-1640 medium (w/v, 1.04% RPMI-1640, 3.45% MOPs, pH was adjusted to 7.0) containing a series of concentrations of different antifungal drugs was used. MICs were determined after 24 hours incubation at 35°C. *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 served as quality controls.

ITS- and MLST-based species identification and phylogenetic analysis

C. vulturna strains were streaked and grown on YPD plates. Genomic DNA of single colonies was extracted for PCR analysis. A fragment containing the internal transcribed spacer (ITS), partial 18S small subunit (SSU), and 28S large subunit (LSU) ribosomal sequences were amplified using the primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') / NL4 (5'-GGTCCGTGTTTCAAGACGG-3'). Eight genes (*AAT1, ACC1, ADP1, ALA1, ERG11, RPB1, RPB2,* and *ZWF1*) were chosen for MLST analysis based on prior studies (3, 4). The following primers were used in the PCR reactions:

AAT1 (540 bp):

AAT1F: aaggagtacacgggtatcac, AAT1R: aacgagctcgttcaatcttc

ACC1 (515 bp):

CvACC1F: accaacaacaacaactacgc, CvACC1R: ccagccaacttcatgatgaa

ADP1 (499 bp):

ADP1F: ttcaaaaagaccaccacgag, ADP1R: acactctccaccggtaatgt

ALA1 (530 bp):

ALA1F: tgcgaactcccaaaagtgta, ALA1R: tttcaaaacccataccggtg *ERG11* (562 bp): ERG11F: aactctcgtttgatggagca, ERG11R: aatgcaacaagaaccaagca *RPB1* (516 bp): RPB1F: agaagagatttaatgcggtg, RPB1R: ccatgtatgtagcaacgtga *RPB2* (513 bp): RPB2F: atcgaggagaaggtggagaa, RPB2R: ttcctcaacacagggcttca *ZWF1* (503 bp): ZWF1F: actcgtctatcctgaaggtg, CvZWF1R: tctcgtcgtcaaggtagga

The PCR products were sequenced and analyzed. The homologous sequences of representative species of the CTG clade were retrieved from the NCBI GeneBank or CGD (http://www.candidagenome.org/) databases. The sequences of *C. vulturna* isolates and other CTG species were analyzed using software mafft v7.015b (5). The phylogenetic tree was generated using the programme RAxML v7.3.2 (6). The General Time Reversible (GTR) model, gamma distribution, and 1000 bootstraps were adopted.

Sequence information for strains CVDH01-CVDH19.

The internal transcribed spacer (ITS) and partial ribosomal sequences for CVDH01-CVDH19 were amplified by PCR using the primer pair ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') / NL4 (5'-GGTCCGTGTTTCAAGACGG-3'). The sequences are listed below. The ITS and partial ribosomal sequences for strains CVDH01-CVDH19 (the sequences were the same):

GCGGAAGGATCATTAAAATAACACTTACACACTGATTTTGACTAGTAAATAA CCCACCAGTTAAGTTCAATTACACAATTAGTAAAACTTTCAACAACGGATCTCTTGG TTCTCGCATCGATGAAGAACGCAGCGAAATGCGATACGTAGTATGACTTGCAGACG TGAATCATCGAATCTTTGAACGCACATTGCGCCTTGGAGCATTCTCCAAGGCATGCC TGTTTGAGCGTGATTTCTTCTCACCGCACCCGGTGGTTTTGCATCCGCGCTAAATATC ATTCCAGCAGCGAAGTCTACGCTTTCACTGCTCCATGCTATTTCCTCAAATCAGGTA GGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGG ATTGCCTCAGTAACGGCGAGTGAAGCGGCAAGAGCTCAACTTTGGAATCGCTCCGG CGAGTTGTAGTCTGGAGGCGGCCGGTCCGCCTTGCGCAACCAAATCTAAGTCCTCTG GAACGAGGCGCTTGAAAGGGTGACAGCCCCGTGGATTTGTCTGTTGTGCTTGGCCCC CCATCTAAAGCTAAATACCGGCGAGAGACCGATAGCGAACAAGTACAGTAATGGAA AGATGAAAAGCACTTTGAAAAGAGAGTGAAACAGTACGTGAAATTGTTGAAAGGGA AGGGCTTGCAGGTAGACAACTGTCAGCATCGGGTGGAGTGGAGCTAGAAGTGGGCG CTGATGTAGCAACTTCGGTTGCATTATACAACGCTCAGATAGCTCCCGTTTCGCCCG AGGATCGCCTTTTGAAGGATG

DNA sequences for MLST analysis (eight genes: *AAT1, ACC1, ADP1, ALA1, ERG11, RPB1, RPB2,* and *ZWF1*)

AAT1 sequence for strains CVDH01-CVDH19:

CGGTTCCAAGACCTACCAGGACGCGGTCAAGAACTTCATTTTCAACAACTCT GACAAGGACACCAACGGTGCTCAATTGATCAAGGACGGCCGTATTGTCACTGCCCA AACCATCTCCGGTACCGGTTCTCTCCGTGTTATCGCCGATTTCCTCAACAGATTCTAC TCCTCGGGTCAGATCATCGTTCCTAAGCCAACCTGGGCTAACCACGTCGCTGTGTTC ACCGATGCTGGTATGAAGGCTGACTTTTACGCCTACTACGACAAGGAGAACAATGG CTTGGACTTTGAGAATCTCAAGAAGTCTGTCGCTGCTGCTGCTGCTGAGGAGTCTGTGAT CTTGTTGCACGCCTGTTGCCACAACCCTACTGGTATGGACTTGACTCCCCAGGAATG GGAGGAGGTTTTGGAGATCATCCAGCAGAAGAAGCTCTTCCCTCTTGTGGACATGGC CTACCAGGGCTTCGCCTCCGGTAACACCTACGAGGACATTGGCTTGATCA

ACC1 sequence for strains CVDH01-CVDH19:

CAATGTCGAGTTGATTGTCGAAATCGCAGAGAGAACCAATGTCCACGCCGTG TGGGCCGGCTGGGGCCACGCCTCGGAAAACCCCATTTTGCCCGAGATGTTGGCCGCC CTGCCCAAAAAATCGTGTTTATCGGCCCGCCAGGCTCCGCCATGAGGTCCTTGGGT GACAAGATCTCCTCCACAATCGTTGCACAGCACGCCGACGTGCCCTGTATCCCCTGG TCCGGTACGGGCGTGCTGGACGTTGAAATTGACAACGAAACGAAATTGGTCTCGGT GTCCGAAGAGACTTACGCCAAGGGCTGCTGCACGAGTCCGGAAGACGGCTTGGAAA AAGCCCGCCAGATCGGTTTCCCCGTCATGATCAAGGCCTCCGAGGGTGGAAGACGGCT AAAGGTATCCGTAAGGTCGACAACGAAGACGATTTTATCTCCTTGTACAAGCAGGCT GCTAACGAGATCCCTGGCTCTCCAATT

ADP1 sequence for strains CVDH01-12 and CVDH 14-19:

ADP1 sequence for strains CVDH13:

ALA1 sequence for strains CVDH01-19:

ERG11 sequence for strains CVDH01-19:

GAAGAAGTTCGCAAAGACAGCCTTGACCAAGGAAGCTTTCCAAAGATACGTC CCTAGAATCCAGGAGGAGGTGTTGGACTACTTCAAGACCTGTCCTGAGTACGAGAT GAACGAGCGCAACAACGGTGTTGCCAACGTGATGAAGACCCAGCCTGAGATGACCA TCTTGACTGCTTCCAAGTCTTTGATGGGCGACGACATGAGAGCCAAGTTTGATGCCT *RBP1* sequence for strains CVDH01-19:

RPB2 sequence for strains CVDH01-19:

GCCCTCGGTGTTGTCCCTGATGGTGATATCTTGGAGCACATTTGTTACGACGCTAAT GACTGGCAAATGTTGGAGATGT

ZWF1 sequence for strains CVDH01-19:

GAAGAGTGAGAGTCATTGTCGAGAAGCCCTTCGGCCACGATTTGGAGTCTTC CAGACAATTGCAGAAAGATTTGGCTCCTCTTTTCACTGAGGAAGAATTGTACAGAAT TGACCACTACTTGGGCAAGGAAATGGTGAAGAACTTGTTGGTGTTGCGTTTTGGTAA TGAGTTATGGTCTGGTGTGTGGGAACAACAGGCATATTTCCTCGGTCCAGATTTCCTTT AAAGAGGCATTCGGAACAGAGGGAAGAGGCGGCTACTTTGACCTGATCGGCATAAT CAGAGACGTCATGCAGAACCACTTATTGCAGGTGTTGACCCTTTTGACCATGGAGAG ACCTGTGTCGTTCGACCCAGAGGCTGTGAGAGATGAAAAGTGAAGGTGCTCAAGG CTTTTGACGATTTTAACCCCAACGACATCTTGCTCGGTCAATATGGTAAGTCTGAAG ATGGCTCTAAGCC

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						Strain collection	Time of	
		Age,			Specimen	date (Days after	admission,	Patient
Case	Sex	у	Diagnosis	Facility type	source	admission)	Days	outcome
C1	Male	45	Hypertension (stage III)	Neuroscience	Blood	Jan 17, 2019	Jan 1, 2019 –	Routine
			and Brain surgery	ward		(16 days)	Feb 12, 2019	discharge
							(42 days)	
C2	Male	60	Traumatic subarachnoid	Neuroscience	Blood	Jun 20, 2019	May 31, 2019	Routine
			hemorrhage tSAH,	ward		(20 days)	- Sep.11,	discharge
			intravetricular				2019	
			hemorrhage, scalp				(103 days)	
			laceration, traumatic					
			pneumonia					
C3	Male	57	Chronic bronchitis,	ICU	Blood	Aug 3, 2019	May 14, 2019	Discontinued
			pulmonary infection			(81 days)	– Aug 8, 2019	care
							(86 days)	
C4	Male	73	Chronic cough and	ICU	Blood	Aug 13, 2019	Aug 10, 2019	Discontinued
			expectoration,			(3 days)	– Sep 2, 2019	care
			Hypertension (stage III)				(23 days)	
C5	Male	43	Injuries to the spleen, rib	General	Blood	Aug 16, 2019	Jul 9, 2019 –	Routine
			fractures	surgery ward		(38 days)	Nov 16, 2019	discharge/
							(130 days)	self care
C6	Male	78	Acute abdominal	ICU	Blood†	Aug 18, 2019	Aug 5, 2019 –	Routine
			disease, Hypertension		and PICC	(13 days)	Sep 21, 2019	discharge
			(stage I)		tip		(47 days)	
C7	Female	16	Injuries to the head, face	ICU	Blood	Aug 27, 2019	Aug 20, 2019	Routine
			are, chest and			(7 days)	– Dec 26,	discharge/
			abdominal tissues				2019	self care
			caused by car accident				(128 days)	
C8	Male	73	Thoraco-	ICU and	Blood	Sep 21, 2019	Sep 7, 2019 –	Routine
			abdominal and pelvic inj	Neuroscience		(14 days)	Nov 6, 2019	discharge/
				ward			(60 days)	self care

Appendix Table. Detailed information on the patients with *C. vulturna* infections*

						Strain collection	Time of	
		Age,			Specimen	date (Days after	admission,	Patient
Case	Sex	У	Diagnosis	Facility type	source	admission)	Days	outcome
			uries caused by car					
			accident					
C9	Male	46	Traumatic epidural	ICU	Blood	Oct 11, 2019	Oct 2, 2019 –	Routine
			hematoma, scalp injury			(9 days)	Jan 14, 2020	discharge/
			and skull fracture				(104 days)	self care
C10	Male	66	Trigeminal neuralgia,	ICU	Blood†	Oct 12, 2019	Jul 19, 2019 –	Routine
			hypertension		and PICC	(73 days)	Nov 14, 220	discharge
					tip		(118 days)	
C11	Female	13	Serious intracranial	ICU	Blood†	Nov 1, 2019	Oct 21, 2019	Routine
			injury, multiple tissue		and PICC	(11 days)	– Jan 13,	discharge
			injuries caused by car		tip		2020	
			accident				(84 days)	
C12	Male	71	Bile duct cancer	General	PICC tip	Nov 19, 2019	Oct 20, 2019	Routine
				surgery ward		(30 days)	– Dec 13,	discharge
							2019	
							(54 days)	
C13	Male	78	Periodic fever	General	Blood	Dec 28, 2019	Nov 26, 2019	Routine
				Medicine ward		(32 days)	– Jan 20,	discharge
							2020	
							(55 days)	
C14	Male	83	Chronic obstructive	ICU	PICC tip	Sep 18, 2020	Aug 30, 2020	Discontinued
			pulmonary disease,			(19 days)	– Sep 22,	care
			lower respiratory tract				2019	
			infection, hypertension				(23 days)	
			(stage III)					
C15	Male	66	Paraplegia, type 2	ICU	Blood	Nov 18, 2020	Oct 30, 2020	Discontinued
			diabetes, pulmonary and			(19 days)	– Dec 15,	care
			urinary tract infections				2020	
							(46 days)	
C16	Male	63	Consciousness Disorder,	ICU	Blood	Feb 11, 2022	Dec 30, 2021	Expired
			Septic shock, sepsis,			(43 days)	– Mar 6, 2022	
			pulmonary and urinary				(66 days)	
			tract infections					
C17	Male	57	Injuries to the head	ICU	PICC tip	Jun 30, 2022	May 26, 2022	Routine
			caused by car accident			(35 days)	– Jul 13, 2022	discharge
							(48 days)	

						Strain collection	Time of	
		Age,			Specimen	date (Days after	admission,	Patient
Case	Sex	У	Diagnosis	Facility type	source	admission)	Days	outcome
C18	Male	66	Advanced gastric	Internal	Blood†	Jul 24, 2022	Jul 7, 2022 –	Routine
			cancer, bladder cancer	Medicine -	and PICC	(17 days)	Aug 30, 2022	discharge
				Oncology	tip		(54 days)	
C19	Male	60	Intracranial infection	Neuroscience	Blood	Aug 26, 2022	J Jul 25, 2022	Routine
				ward		(32 days)	– Oct 26,	discharge
							2022	
							(94 days)	

*PICC, peripherally inserted central catheter; ICU, intensive care unit.

†When *C. vulturna* was isolated from two or more specimen sources, strains isolated from the blood samples were used for biological and DNA sequencing analyses.



Appendix Figure 1. Monthly incidence of *C. vulturna* infections in a Shanxi, China hospital. C1–C19, patient cases 1 to 19. Only two cases of *C. vulturna* infections were found during the peak COVID-19 period, January 1, 2020–January 1, 2022, perhaps because of the enhanced hygiene measures implemented.



Appendix Figure 2. Maximum-likelihood phylogeny analysis of the CTG clade species based on the internal transcribed spacer and partial ribosomal sequences. The tree was generated using the program RAxML (https://cme.h-its.org/exelixis/web/software/raxml). The general time reversible model model, gamma distribution, 1,000 bootstraps, and midpoint root were adopted. Strain sequence information: *C. haemulonii* (CBS10969, JX459773.1), *C. haemulonii var. vulneris* (CBS12439, MK394151.1), *C. pseudohaemulonii* (CBS10004, MK394152.1), *C. duobushaemulonii* (CBS7798, MK394153.1) from the NCBI GeneBank; SRR11091965–67, SRR11092032, SRR11092036, and SRR22996287 from the NCBI WGS database; *C. auris* (B8441), *Clavispora lusitaniae* (ATCC42720), *C. parapsilosis* (CDC317), *C. orthopsilosis* (Co90–125), *C. tropicalis* (MYA3404), *C. albicans* (SC5314), *C. dubliniensis* (CD36) from the CTG database (http://www.candidagenome.org).



Appendix Figure 3. Biofilm morphologies of *C. vulturna* and *C. auris* isolates. *C. vulturna* strains are CVDH01, CVDH03–05, CVDH07–17, and CVDH19. *C. auris* strains are BJCA001 and SJ-01. Biofilms were developed on silicone squares at 30°C for 24 hours. Lee's glucose medium was used for biofilm growth. Compared to the other *C. vulturna* isolates, strains CVDH07, CVDH12, and CVDH13 developed weaker biofilms on the silicone squares. *C. auris* strain SJ01 developed robust biofilms, whereas *C. auris* strain BJCA001 formed comparatively weaker biofilms. This figure is associated with Figure 2 in the main article.