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# Rapid Detection of *ERG11*-Associated Azole Resistance and *FKS*-Associated Echinocandin Resistance in *Candida auris*

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**ABSTRACT** *Candida auris* is an emerging multidrug-resistant yeast that can cause serious invasive infections. The accurate and rapid assessment of antifungal resistance is important for effective patient management. A novel and highly accurate diagnostic platform was established for the rapid identification of *ERG11* mutations conferring azole resistance and *FKS1* mutations associated with echinocandin resistance in *C. auris*. Using allele-specific molecular beacons and DNA melting curve analysis following asymmetric PCR, a duplex *ERG11* assay and a simplex *FKS1* HS1 assay were developed to identify the most prominent resistance-associated mutations (Y132F and K143R in *ERG11*; S639F in *FKS1* HS1) within 2 h. Assays were validated by testing a panel of 94 *C. auris* clinical isolates in a blind manner. The molecular diagnostic results from the assays were 100% concordant with DNA sequencing results. This platform has the potential to overcome the deficiencies of existing *in vitro* susceptibility-based assays to identify azole- and/or echinocandin-resistant *C. auris*, and thus, it holds promise as a surrogate diagnostic method to direct antifungal therapy more effectively.

**KEYWORDS** *Candida auris*, *ERG11*, *FKS*, azole, echinocandin, molecular beacons, resistance

**C**andida auris is an emerging yeast pathogen that has posed a serious challenge to global health. *C. auris* was first identified in 2009 from the external ear canal discharge of a patient in Japan (1) and quickly spread around the globe, with reports from five continents (2, 3). *C. auris* can cause invasive infections and has been associated with outbreaks in health care settings (4). The U.S. Centers for Disease Control and Prevention (CDC) issued a clinical alert to health care facilities about *C. auris* to local or public health authorities in June 2016 (5). Moreover, many clinical isolates of *C. auris* have been reported to be multidrug resistant (MDR) with reduced susceptibility to two or even three classes of antifungal drugs, exhausting available therapeutic options (6–8).

Despite multiple mechanisms of azole resistance being described in other *Candida* species, mutations in *ERG11* (the gene encoding lanosterol 14-$\alpha$-demethylase, the target enzyme in the ergosterol synthesis pathway) have been the only reported mechanism responsible for azole resistance in *C. auris* thus far. A number of *ERG11* mutations have been observed in *C. auris* isolates originating from different geographic areas (7, 9); however, only the Y132F and K143R substitutions are definitely linked with the reduced azole susceptibility in *C. auris* (7). Of note, the direct contribution of Y132F and K143R to conferring azole resistance in *C. auris* has been confirmed in a recent study that expressed different *C. auris ERG11* alleles in *Saccharomyces cerevisiae* (10). Therefore, these two substitutions have been suggested to be valuable as initial

markers for *C. auris* azole resistance within South Asian and South American clade isolates (10).

Echinocandin resistance, although at a lower frequency than azole resistance, has been reported in isolates from both India and South Africa (9, 11–13). As in other *Candida* species, echinocandin resistance in *C. auris* is associated with hot spot (HS) mutations of *FKS*, the gene encoding the catalytic subunits of the drug target enzyme $\beta$-1,3-D-glucan synthase (14). In an Indian study, all four isolates containing a serine to phenylalanine amino acid substitution at codon 639 (S639F) had elevated pan-echinocandin MICs (7) and did not respond to echinocandin therapy in the mouse model of invasive candidiasis (6). A different amino acid substitution in the same position (S639P) was recently reported in echinocandin-resistant *C. auris* isolates (13).

Given the prevalence of MDR *C. auris*, resistance surveillance is recommended in patients who are infected or colonized with *C. auris* and are on antifungals (15). Antifungal susceptibility profiles can be assessed phenotypically, using either microdilution or disk diffusion MIC assays in accordance with the Clinical and Laboratory Standards Institute (CLSI) M27-A3 standard (16) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) definitive document EDef 7.2 (17). However, standardized susceptibility assays have limitations, such as being time-consuming, subjective for reading visual end points, and prone to interlaboratory variability, especially for caspofungin. They also show known Eagle effects with echinocandins, which interfere with accurate MIC readings (6).

Now that azole and echinocandin resistance in *C. auris* are closely associated with specific *ERG11* and *FKS1* mutations, genotyping both genes is likely to offer the most valuable information to facilitate effective resistance detection and antifungal therapy. Recently, we developed a rapid diagnostic platform to identify *FKS*-associated echinocandin resistance in *Candida glabrata*, using asymmetric PCR in conjunction with molecular beacon (MB) probe-based melting curve analysis (18). Herein, we have adopted the same strategy to develop a molecular diagnostic assay platform for the rapid genotyping of *ERG11* and *FKS1* HS1 of *C. auris*. Assay performance was evaluated in a blind manner on a panel of clinical *C. auris* isolates acquired from different sources.

## RESULTS

**Melting curve analysis-based *ERG11* genotyping.** To distinguish azole-resistant *ERG11* mutants from the wild type (WT), we designed two molecular beacon probes labeled with different fluorophores (6-carboxyfluorescein [FAM] and CAL Fluor Red [CR610]) to facilitate one-step genotyping at both target amino acid positions Y132 and K143. The probes were designed to perfectly match the Y132WT- and K143R-encoding DNA sequences in the target region but possess lower binding energies to non-Y132WT and K143WT sequences, respectively. To enable the efficient dissociation of probe-target hybrids for rapid genotyping, asymmetric PCR amplifications were optimized to generate an excess of the antisense strand of the target. Owing to different thermaldynamic stabilities of the probe-target hybrids, characteristic profiles were produced for different *ERG11* genotypes in the subsequent melting curve analysis. Figure 1A shows representative melting curves of the Y132 genotypes. The Y132F profile had a signature melting temperature ($T_m$) of 55.84°C $\pm$ 0.07°C, remarkably different from the $T_m$ of 60.53°C $\pm$ 0.07°C of the WT. The K143 profile was captured simultaneously in the same assay through the CR610 channel. A melting curve with the $T_m$ at 52.48°C $\pm$ 0.04°C was featured for the WT, and the $T_m$ was obtained at 58.90°C $\pm$ 0.06°C for K143R (Fig. 1B).

**Melting curve analysis-based *FKS1* HS1 genotyping.** In the *FKS1* HS1 assay, an MB complementing the WT sequence in the target region was used to distinguish the mutated genotype from the WT on the postamplification melting curve. Figure 1C shows melting curves of *FKS1* HS1 WT and mutant *fks1* encoding S639F from representative strains. The signature melting temperature for each *FKS1* HS1 genotype was widely separated from each other, with an approximate 6°C difference.

**Probe validation experiments.** As a proof of concept, we evaluated the diagnostic performance of this novel genotyping platform using a panel of 94 *C. auris* clinical

## (A) *ERG11* Y132 melting curve



## (B) *ERG11* K143 melting curve



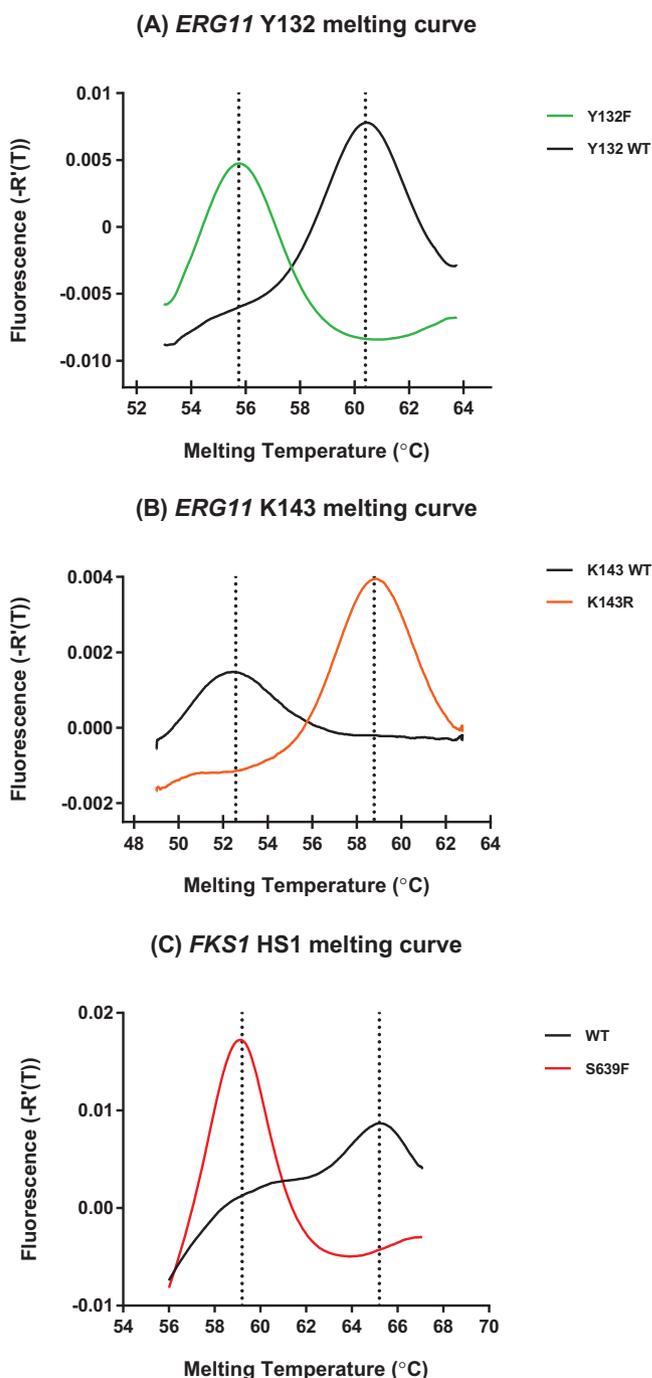## (C) *FKS1* HS1 melting curve



**FIG 1** Representative melting profiles for *C. auris* *ERG11* genotypes, including Y132WT and Y132F (A) and K143WT and K143R (B) and *FKS1* HS1 genotypes, including WT and S639F (C).

isolates. Assay performers were blinded to information on the *ERG11* and *FKS1* sequences and antifungal susceptibility at the time of molecular diagnosis. All isolates were tested in duplicate for these assays, and reference strains representing all genotypes used for platform establishment were tested in parallel in every run. The inclusion of reference strains in each test run was important, as they served as systematic quality controls to ensure the assay's accuracy and reproducibility. Robust and reproducible melting profiles were observed for all tested isolates. The *ERG11* duplex assay identified 73 Y132WT sequences ($T_m$ = 60.38°C ± 0.08°C), 21 Y132F mutations (55.74°C ± 0.04°C) (Fig. 2A), 78 K143WT sequences ($T_m$ = 52.56°C ± 0.13°C), and 16 K143R mutations
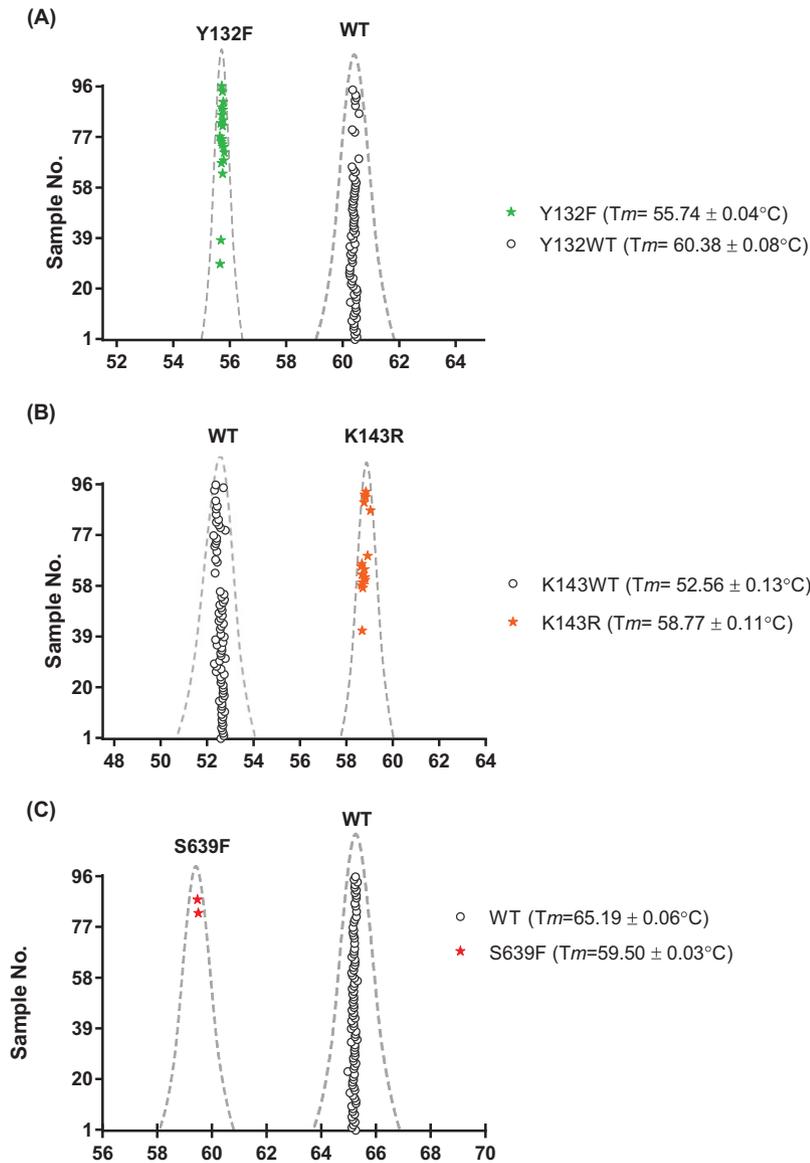
FIG 2 *ERG11* Y132 (A), *ERG11* K143 (B), and *FKS1* HS1 (C) $T_m$ distributions of 94 *C. auris* clinical isolates tested in a blind manner.

(58.77°C ± 0.11°C) (Fig. 2B). The *FKS1* HS1 assay picked up 92 WT sequences ($T_m$ = 65.19°C ± 0.06°C) and captured two S639F alleles ($T_m$ = 59.50°C ± 0.03°C) (Fig. 2C). DNA sequencing results confirmed all predicted genotypes. In summary, both *ERG11* and *FKS1* HS1 assays are highly accurate in differentiating prominent azole- and echinocandin-resistant mutants from the WT population, with 100% specificity and 100% sensitivity. The assays in this study are designed to be specific to *ERG11* or *FKS1* HS1 of *C. auris*; we tested closely related species and no cross-reactivity to any other species was detected.

## DISCUSSION

*C. auris* has become a global concern, with existing multidrug resistance to fluconazole and amphotericin B, and is characterized by a strong potential for nosocomial transmission (4, 9). Studies examining the susceptibility of this organism to antifungals have used a variety of methods, including CLSI and EUCAST broth microdilution (16, 17) or their commercial counterparts, such as Sensititre YeastOne, Etest and the Vitek 2 yeast susceptibility system. At present, there are no antifungal clinical breakpoints

reported for *C. auris* by CLSI or EUCAST. The tentative epidemiological cut off values (ECVs) proposed in a recent study were determined based on the analysis of MIC distributions of 123 clinical isolates of *C. auris* from India (19). These ECVs are valuable in the analysis of MICs of isolates from the South Asian clade, yet application of the ECVs to isolates from other clades may lead to incorrect estimation of resistance, since there are strong indications that MIC distributions can vary substantially for *C. auris* isolates from different clades. Therefore, an analysis of susceptibility results obtained by these phenotypic tests is not straightforward and may have difficulties defining susceptible or resistant isolates.

There is an emerging need for alternative diagnostic tools, which allow rapid and accurate identification of resistance-associated mutations in *C. auris*. These tests have the potential to become part of the routine clinical laboratory tests for resistance detection to help direct therapy and enhance epidemiological surveillance. Encoding the primary drug targets of which specific mutations account for the most prominent azole or echinocandin resistance in *C. auris*, *ERG11* and *FKS1* are perfect targets for the molecular diagnosis of drug-resistant *C. auris*. Along this line, we developed the first molecular diagnostic platform for rapid *ERG11* and *FKS1* HS1 genotyping of *C. auris* isolates. The assays are designed to detect known mechanisms of resistance and are not intended at this stage to be a surrogate for the standard antifungal susceptibility test. This platform, taking advantage of the single nucleotide polymorphism sensitive nature of molecular beacons and incorporating asymmetric PCR, accurately discriminated WT from drug-resistant *ERG11* and *FKS1* HS1 mutants within 2 h, without the need of DNA sequencing. Both *ERG11* and *FKS1* HS1 assays are highly robust, as each genotype presented a signature melting curve with a $T_m$ highly distinguishable from others. Bearing a robust discrimination power, this novel platform demonstrated 100% accuracy in identifying *C. auris* clinical isolates in a blind manner as either WT or drug resistant genotype in the subsequent proof-of-concept diagnostic performance evaluation.

There are some limitations in this study. First, only Y132 and K143 were included in the *ERG11* genotyping assay. We recognize the possibility of other mechanisms being involved in azole resistance in *C. auris*. However, given that Y132F and K143R are currently the most prevalent and the only mutations confirmed to confer azole resistance in *C. auris*, the present *ERG11* assay is of proper clinical value. Second, there was only one mutated genotype (S639F) included in the *FKS1* HS1 assay development and evaluation. To date, only two *FKS1* HS1 mutations have been found in echinocandin-resistant *C. auris* isolates around the world, S639F and the recently reported S639P (13). It is unfortunate that we did not have the S639P mutant strain encompassed in our study due to limited strain access. There is a chance that the $T_m$ value of the S639P allele may not be distinguishable from S639F, since these mutations occur on the same codon and their melting profiles are presumably similar. However, it is anticipated that the S639P melting signature will be remarkably different from that of the WT, given what has been generated with the S639F allele and our previous experience with *C. glabrata FKS* alleles (18), ensuring that the assay is a useful tool for discriminating echinocandin-resistant mutants from the susceptible WT population. Nevertheless, further work to include the S639P allele is warranted upon availability of the strain. It should be noted that the *FKS1* HS1 assay in this study is an open platform that allows the easy incorporation of any unknown HS1 alleles without the need of assay redesign. The assay becomes applicable to any novel *FKS1* HS1 mutations as soon as the corresponding genotype is captured and added into the reference genotype library.

In summary, we have developed a rapid and accurate diagnostic platform that has the potential to overcome the deficiencies of existing MIC-based assays to identify azole- and echinocandin-resistant *C. auris*. It holds the promise to be a robust diagnostic method to better detect resistance-associated mutations and to direct more effective antifungal therapy against infections caused by *C. auris*, and it can be used for epidemiologic surveillance.

**TABLE 1** Distribution of different *Candida auris* EGR11 and *FKS1* HS1 variant MICs

| Gene variant | MIC range (mode) (mg/liter) for: | | | | |
|---|---|---|---|---|---|
| | Fluconazole | Voriconazole | Micafungin | Anidulafungin | Caspofungin |
| *ERG11* substitutions | | | | | |
| WT | 2 to 64 (2) | 0.03 to 1 (0.06) | | | |
| Y132F | 64 to >128 (>128) | 2 to >16 (4) | | | |
| K143R | 32 to >128 (>128) | 0.12 to 1 (0.5) | | | |
| | | | | | |
| *FKS1* HS1 substitutions | | | | | |
| WT | | | 0.03 to 0.5 (0.125) | 0.03 to 1 (0.5) | 0.125 to 1 (0.25) |
| S639F | | | 16 | 4 to >16 (>16) | 8 to 16 (8) |

## MATERIALS AND METHODS

**Candida auris strains and culture conditions.** A total of 98 *C. auris* isolates were included in the present study. Forty *C. auris* isolates were obtained from Vallabhbhai Patel Chest Institute, University of Delhi (Delhi, India); 48 isolates were obtained from Clinica General del Norte (Barranquilla, Colombia); 8 isolates were obtained from a clinic of high complexity (Santa Marta, Colombia); and 2 isolates were from Westerdijk Fungal Biodiversity Institute (formerly CBS-KNAW, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands). Four strains with different *ERG11* (Y132 and K143) and *FKS1* HS1 (S639) genotypes were used for assay development and reference library establishment, and the remaining 94 *C. auris* isolates were used for the validation study in a blind fashion. Strains were grown on yeast extract-peptone-dextrose (YPD) agar plates, and species identification of all isolates was performed by sequencing of the ribosomal DNA (rDNA) region (6).

**Antifungal susceptibility testing.** Echinocandin (caspofungin, micafungin, and anidulafungin) and azole (fluconazole and voriconazole) MICs were determined according to CLSI protocol M27-A3 (Table 1) (16).

**DNA extraction.** *C. auris* DNA was prepared by incubating a single colony of a strain in 100 $\mu$l of extraction buffer (60 mM NaHCO$_3$, 250 mM KCl, 50 mM Tris; pH 9.5) at 95°C for 10 min, followed by an addition of 100 $\mu$l of 2% bovine serum albumin (20).

**Primers and probe design.** Two sets of asymmetric PCR primers were designed to amplify an F132- and K143-containing region of *ERG11* and the *FKS1* HS1 region of *C. auris*. The *ERG11* region was amplified by using excess primer CauERG11-X (5'-ACTTCCTCTTGGATTCTGGG-3') and limiting primer CauERG11-L (5'- TGCTTATTCCCACTTGACCACTCCAG-3'). Two molecular beacons were designed targeting the Y132WT and K143R allele sequences of *ERG11*, and they were CauERG11-Y132WT (5'-FAM-<u>CGCGAT</u>TGTCATTTACGACTGTCCC<u>ATCGCG</u>-DABCYL-3', where DABCYL is *N*-[4-(4-dimethylamino)phenylazo]benzoic acid) and CauERG11-K143R (5'-CR610-<u>CGCGATA</u>TGGAGCAGAGG AAATTTG<u>ATCGCG</u>-BHQ1-3' [BHQ1, black hole quencher 1]). The excess primer for *FKS1* HS1 amplification was CauF1H1-X (5'-CGTCATGGTGGACAAGTTTCTA-3') and the limiting primer was CauF1H1-L (5'-GGGTCACTGTGTTTGCTGCTAAGTTGG-3'). The *FKS1* HS1 MB was CauF1H1-WT (5'-FAM-<u>CGCGAC</u>TTCTTGACTTTGTCCTTGAGAGATCCT<u>GTCGCG</u>-DABCYL-3') (underlining signifies the stem portion of the molecular beacon).

**Asymmetric PCR and molecular beacon-based melting curve analysis.** Asymmetric PCR was carried out on the Mic real-time PCR cycler (Bio Molecular Systems) in a 10-$\mu$l reaction volume using SensiFAST probe no-ROX mix (Bioline, London, UK). The *ERG11* duplex assay contained 800 nM CauERG11-X, 40 nM CauERG11-L, 500 nM CauERG11-Y132WT MB, 500 nM CauERG11-K143R MB, and 10 to 25 ng of DNA template. *ERG11* PCR conditions were 95°C for 3 min; 45 cycles of 10 s at 95°C, 20 s at 60°C, and 30 s at 72°C; and 2 min at 72°C. Immediately after amplification, melting curve analysis was initiated as a minute incubation at 95°C, after which it was melted from 53°C to 64°C with a ramp rate of 0.1°C/s for the Y132WT probe and melted from 49°C to 63°C with a ramp rate of 0.1°C/s for the K143R probe. The *FKS1* HS1 assay contained 1 $\mu$M CauF1H1-X, 40 nM CauF1H1-L, and 500 nM CauF1H1-WT MB. *FKS1* HS1 PCR thermal cycling consisted of a 3-min incubation at 95°C; 45 cycles of 10 s at 95°C, 20 s at 60°C, and 30 s at 72°C; and then incubation at 72°C for 2 min. Immediately after amplification, melting curve analysis was performed at 95°C for 2 min, after which it was melted from 54°C to 67°C with a ramp rate of 0.025°C/s.

**DNA sequencing.** *ERG11* and *FKS1* HS1 regions were amplified and sequenced in both directions as previously described (7).

**Statistical analysis.** $T_m$ values for *ERG11* and *FKS* genotypes were determined by melting curve analysis using the Mic real-time PCR system software (Bio Molecular Systems). Genotyping results by rapid molecular diagnostic assays were compared with the DNA sequencing results. The $T_m$ distribution of clinical isolates was analyzed by GraphPad Prism 7.01 software. The accuracy of the novel assays discriminating WT from mutated *ERG11* and *FKS* genotypes was evaluated by calculating sensitivity and specificity for each assay.

**Accession number(s).** The *ERG11* and *FKS1* sequences of representative strains of each genotype in this study were deposited in GenBank with accession numbers MK059959 to MK059966 and MK059967 to MK059974, respectively (Table S1).

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