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RNA-based antibiotic susceptibility testing of *tmexCD-toprJ*-mediated tigecycline resistance in *Klebsiella pneumoniae*

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Abstract

The emergence and prevalence of plasmid-encoded RND-type efflux pump TMexCD-TOprJ severely compromise tigecycline treatment, which is recognized as the last resort for multidrug-resistant (MDR) Gram-negative bacterial infections. There is an urgent need for rapid antibiotic susceptibility testing (AST) that can simultaneously identify the genotype and phenotype of *tmexCD-toprJ*-positive bacteria. Through characterizing transcriptional profiling responses of *tmexCD-toprJ*-positive and -negative strains after exposure to 2 μg/mL tigecycline, here we identified 12 differentially RNA biomarkers and developed an RNA-based AST (RBAST) to distinguish *tmexCD-toprJ*-positive and -negative *K. pneumoniae*. These mRNA biomarkers were successfully validated in tigecycline exposure time variations, concentration shifts, and other *tmexCD-toprJ* variants. In addition, a group of clinical isolated strains was effectively distinguished using RBAST, with an accuracy of over 94% during 3 h test period. Our work highlights the potential of RNA transcripts as biomarkers for rapid AST, which will contribute to deploying effective antibiotic regimens in clinical practice.

Keywords Antibiotic resistance, *tmexCD-toprJ*, Tigecycline, AST, Bacteria

Introduction

Klebsiella pneumoniae is a type of Gram-negative bacteria causing various healthcare-associated infections, such as respiratory tract infections, pneumonia and bloodstream infections [1]. In recent years, owing to the widespread use and abuse of antibacterial drugs, *K. pneumoniae* has become highly resistant to antibiotics such as β-lactams [2], aminoglycosides [3], and quinolones [4], resulting in clinical treatment failures [5].

Tigecycline is a semi-synthetic glycylcycline, one of the derivatives of minocycline. The antibacterial mechanism of action of tigecycline is like that of early tetracycline antibiotics, which act by binding to 30S ribosome subunit and inhibiting bacterial protein synthesis. Since tigecycline can evade the ribosome protection mechanism and efflux mechanism associated with resistance to other tetracycline antibiotics [6], tigecycline is recognized as the last line of defense for the treatment of multidrug-resistant (MDR) Gram-negative bacterial infections.

With the amount of tigecycline is gradually rising, the problem of drug resistance is becoming increasingly prominent. Heretofore, tigecycline-resistant strains are mainly concentrated in *Enterobacteriaceae*, of which *K. pneumoniae* are the most common species [7–9]. It

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has been found that *tet*(A) gene, which originally mediates bacterial resistance to traditional tetracyclines, was mutated in *K. pneumoniae* and *E. coli*, thus reducing tigecycline activity [10]. From 2019 to 2021, novel tigecycline resistance genes such as *tet*(X4) and its variants were identified in *A. baumannii* and *Enterobacteriaceae* [9, 11, 12]. In addition, plasmid-encoded RND-type efflux pump TMexCD1-TOprJ1, recently discovered in human and animal samples, can reduce the susceptibility of all tetracyclines, including tigecycline [13]. In recent years, other *tmexCD-toprJ* variants, including *tmexCD2-to*prJ2 in *Raoultella ornithinolytica* and *K. pneumoniae* [14], *tmexCD3-toprJ1b* in *Proteus* and *Pseudomonas* [15], *tmexCD4-toprJ4* in *Klebsiella quasipneumoniae* and *Enterobacter roggenkampii* [16], *tmexC3D5-toprJ2b* in *Oceanimonas* [17] and *tmexC6D6-toprJ1b* in *Pseudomonas mendocina* [18], have also been discovered. More worryingly, this novel resistance determinant is not only resistant to multiple drugs, but is also reported to be present in the same plasmid carrying other resistance genes such as *mcr*, bla_{NDM} and *tet*(X) and can co-transfer with them [19, 20]. Therefore, it is urgent to develop a rapid and accurate antibiotic susceptibility testing (AST) to identify *tmexCD-toprJ*-mediated tigecycline-resistant bacteria.

Although AST techniques used in traditional clinical practice, such as disk-diffusion susceptibility testing and the broth microdilution method, have the advantage of low cost and high accuracy, it is labor-intensive and time-consuming [21]. The latest molecular diagnostic techniques, such as microfluidics and nucleic acid amplification assays, have greatly shortened the time to diagnose bacterial infections, but their prospect of ameliorating antibiotic stewardship remains unfulfilled [22]. Although the next sequencing technology has been reduced in cost and has been expanding the pool of known drug resistance, it still cannot provide information on both genotype and phenotype [23]. In contrast, the resistance phenotype of bacterial strains can be quickly differentiated by quantitative assessment of antibiotic-responsive RNA transcripts without regard to the mechanisms of resistance or genetic background [24].

In this study, we developed a specific RNA-based AST (RBAST) to rapidly distinguish tigecycline-susceptible isolates from *tmexCD-toprJ*-mediated tigecycline-resistant isolates in *K. pneumoniae*. After treatment with 2 μg/mL tigecycline for 60 min, differentially expressed genes (DEGs) in *tmexCD1-toprJ1*-negative and -positive strains were initially screened. Quantitative reverse transcription PCR (RT-qPCR) was used to further validate the candidate RNA biomarkers with time-span and tigecycline-concentration changes. The RNA biomarkers screened above were finally validated in other *tmexCD-toprJ* variants and isolates, and the susceptible results were compared with those of traditional AST.

Results

Different transcriptomic responses between *tmexCD1-toprJ1*-positive and -negative bacteria upon tigecycline stimulation

To initially screen out specific RNA transcripts that can effectively identify tigecycline-resistant and -susceptible strains, engineered DH5α-pUC19-*tmexCD1-toprJ1* and DH5α-pUC19 were constructed. After treatment with tigecycline at a breakpoint concentration of 2 μg/mL for 60 min, the transcriptional profiling responses of the above strains were compared using RNA sequencing. Shifts in the transcriptome response of *tmexCD1-toprJ1*-negative bacteria after tigecycline stimulation are shown in Fig. 1A, with 858 genes identified with significant shifts in mRNA expression in a total of 4278 genes. Among them, there were 448 genes significantly upregulated and 410 genes significantly downregulated. Nevertheless, in *tmexCD1-toprJ1*-positive bacteria, mRNA expression of only 143 genes shifted significantly, including 103 upregulated genes and 40 downregulated genes (Fig. 1B). After tigecycline stimulation, only six upregulated genes and seven downregulated genes were shifted simultaneously in both groups. There were 430 upregulated and 401 downregulated genes in *tmexCD1-toprJ1*-negative bacteria, but mRNA expression unshifted in *tmexCD1-toprJ1*-positive bacteria (Fig. 1C). In contrast to the DH5α-pUC19-*tmexCD1-toprJ1* group, more DEGs were found in *tmexCD1-toprJ1*-negative groups after tigecycline exposure. Furthermore, the significantly changed genes ($Log_2FC \leq -2$ or ≥ 2, $P < 0.05$) were selected for additional confirmation. A principal component analysis (PCA) was performed to further explore the difference between *tmexCD1-toprJ1*-positive and -negative bacteria after the transcriptome response change at a breakpoint concentration tigecycline. As shown in Fig. 1D, the tigecycline treatment is very close to the untreated *tmexCD1-toprJ1*-positive bacteria, indicating that the two groups have little difference in gene expression profiles. However, the tigecycline treatment is relatively distant from the untreated *tmexCD1-toprJ1*-negative bacteria, indicating that the expression profiles of the two groups are quite different. These results suggest that after the breakpoint concentration tigecycline treatment, the transcriptome response changes in *tmexCD1-toprJ1*-positive and -negative bacteria are quite different.

Functional enrichment of differentially expressed genes (DEGs)

To gain insight into the transcriptome changes of *tmexCD1-toprJ1*-positive and -negative bacteria after

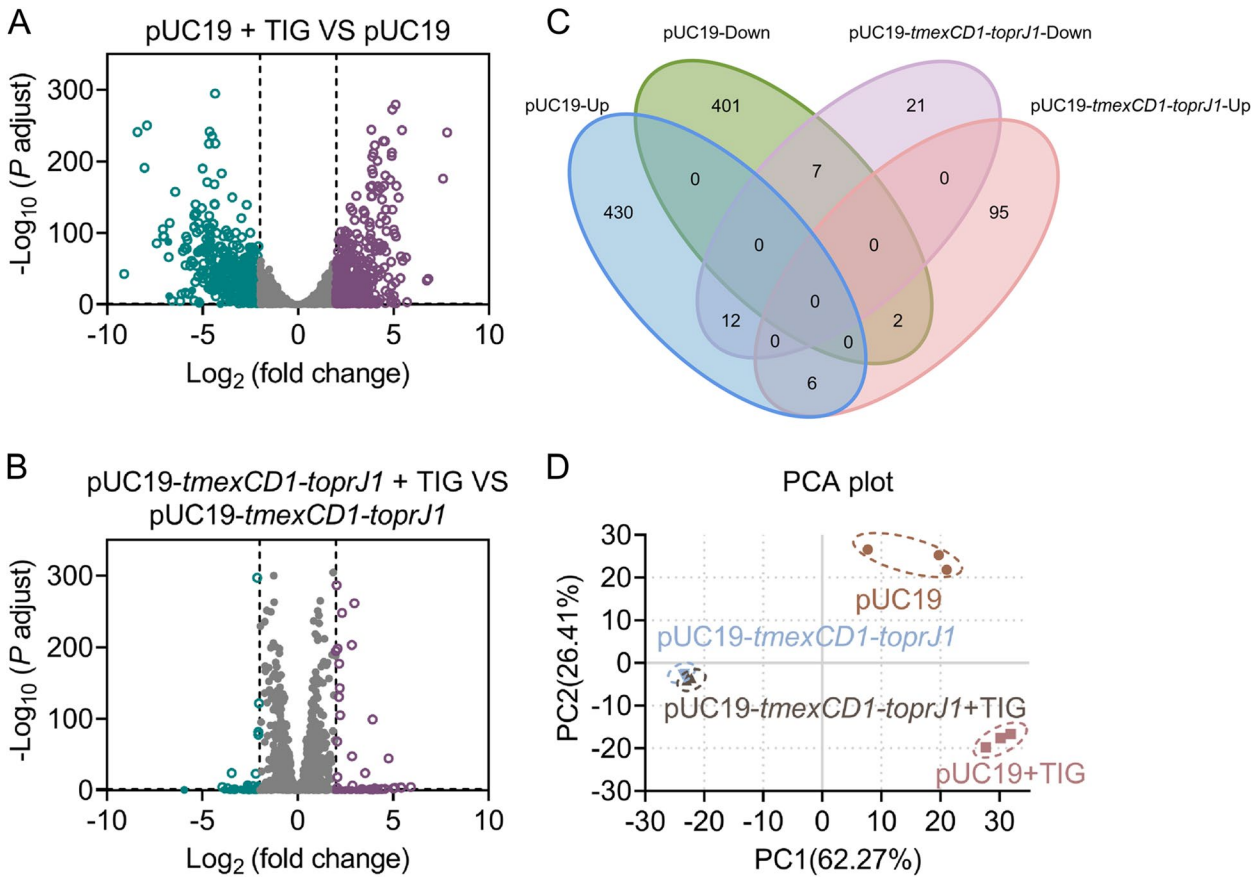

Fig. 1 Differential gene expression of *tmexCD1-toprJ1*-negative and -positive strains upon tigecycline treatment. **A** and **B** Scatter diagram of DEGs from *tmexCD1-toprJ1*-negative tigecycline-susceptible (DH5α-pUC19) and *tmexCD1-toprJ1*-positive tigecycline-resistant (DH5α-pUC19-*tmexCD1-toprJ1*) compared with their own control. Upregulated genes were indicated in purple points, downregulated genes were indicated in green points. **C** Venn diagrams displayed the number of metabolites significantly affected by *tmexCD1-toprJ1*-negative (DH5α-pUC19) and *tmexCD1-toprJ1*-positive (DH5α-pUC19- *tmexCD1-toprJ1*) compared with their own control. FDR < 0.05, $Log_2FC \leq -2$ or ≥ 2 and $P < 0.05$. **D** The first four principal components for metabolite levels from *tmexCD1-toprJ1*-negative (DH5α-pUC19) and *tmexCD1-toprJ1*-positive (DH5α-pUC19- *tmexCD1-toprJ1*) with or without tigecycline exposure were expressed by PCA score plots

2 μg/mL tigecycline stimulation, Gene Ontology (GO) analyses and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were used to functionally annotate DEGs (Fig. 2). Tigecycline inhibits the translation elongation step by binding to the ribosomal 30S subunit A and preventing aminoacyl tRNAs from entering the ribosomal A site. Therefore, in order to combat the inhibition of amino acid synthesis after tigecycline treatment, the upregulated genes of *tmexCD1-toprJ1*-negative bacteria group were mainly enriched in ribosomal small and large subunit assembly, peptide biosynthetic process, cellular protein-containing complex assembly, ribonucleoprotein complex assembly. In addition, the mRNA expression levels in response to antibiotics and in pilus-related pathways were also upregulated. After tigecycline stimulation, the downregulated genes in *tmexCD1-toprJ1* negative bacteria were mainly enriched in the pathways responsible for the catabolism of organic substances such as amino acids, carboxylic acid, nucleobase and oxoacid, as well as the TCA cycle and response to stimulus (Fig. 2A and B). The results of the KEGG analysis also further verify the results of the GO analysis (Fig. 2C and D). The DEGs of *tmexCD1-toprJ1*-positive bacteria in the transcriptome after 2 μg/mL tigecycline-stimulated were also analyzed by GO and KEGG. Upregulated genes were mainly enriched in some amino acid metabolic pathways, and downregulated genes were mainly concentrated in biological adhesion, transport activity, membrane and pH-related pathways (Fig. 2E and F). Since DH5α-pUC19-*tmexCD1-toprJ1* had relatively few genes with mRNA expression changes upon exposure to tigecycline, these genes were also enriched with relatively few pathways. However, the pathway enriched

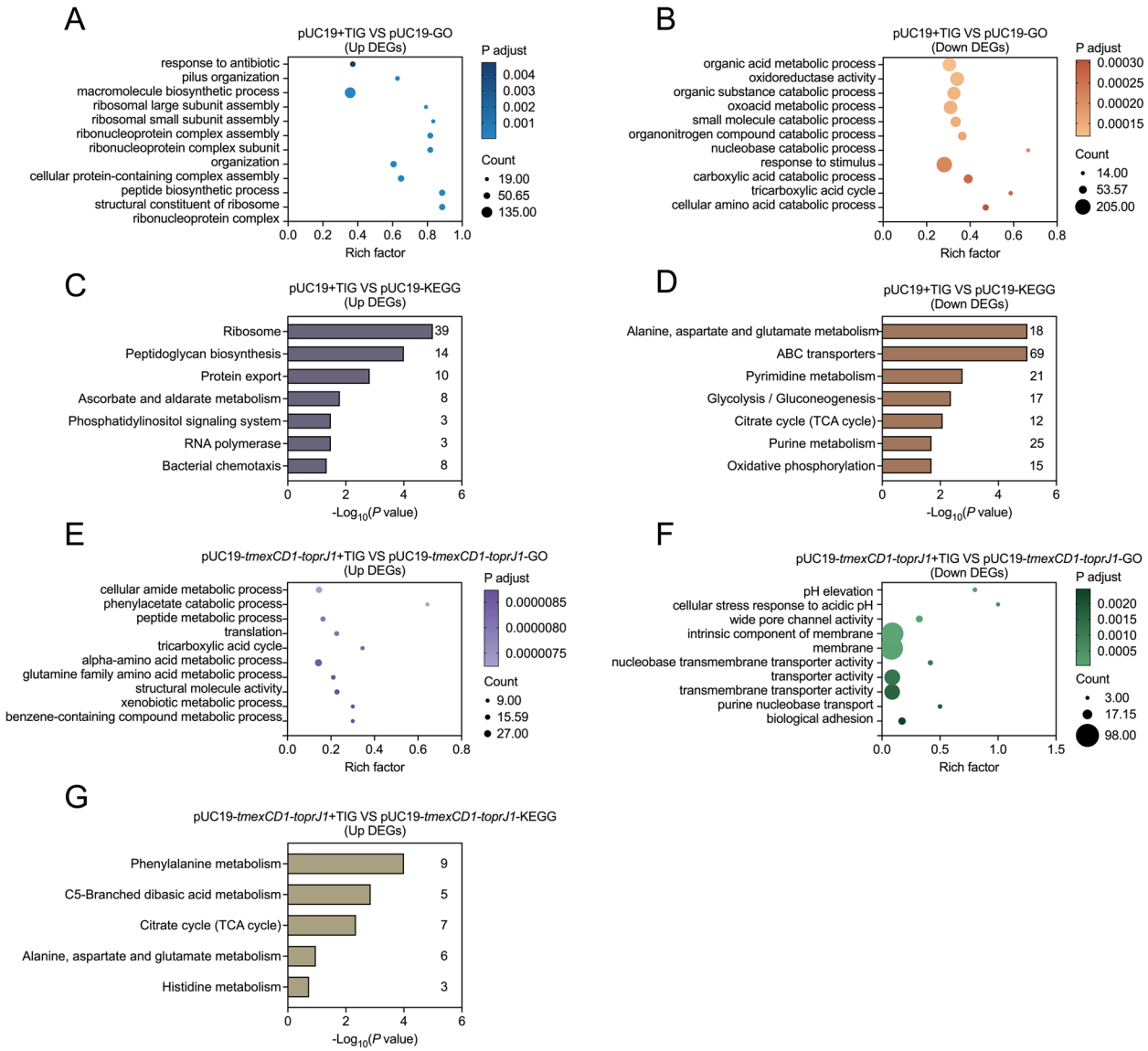

Fig. 2 Functional enrichment of DEGs upon antibiotic exposure. GO pathway enrichment in *tmexCD1-toprJ1*-negative tigecycline-susceptible (DH5α-pUC19) (**A** and **B**) and *tmexCD1-toprJ1*-positive tigecycline-resistant (DH5α-pUC19- *tmexCD1-toprJ1*) (**E** and **F**) group after tigecycline exposure compared with their own control. FDR < 0.05, $Log_2FC \leq -2$ or ≥ 2 and $p < 0.05$. KEGG pathway enrichment in *tmexCD1-toprJ1*-negative tigecycline-susceptible (DH5α-pUC19) (**C** and **D**) and *tmexCD1-toprJ1*-positive tigecycline-resistant (DH5α-pUC19- *tmexCD1-toprJ1*) (**G**) group after tigecycline exposure compared with their own control. FDR < 0.05, $Log_2FC \leq -2$ or ≥ 2 and $p < 0.05$

by the expression of differential genes in the *tmexCD1-toprJ1*-negative bacteria group was largely absent in *tmexCD1-toprJ1*-positive bacteria. In summary, the transcriptome changes of *tmexCD1-toprJ1*-positive and -negative control bacteria are quite different after tigecycline treatment, so it is feasible to determine the antibiotic susceptibility of tested strains based on changes in the expression of certain mRNA biomarkers after antibiotic stimulation at breakpoint concentrations.

Selection of candidate RNA biomarkers

After tigecycline treatment, genes with significant changes ($Log_2FC \leq -2$ or $Log_2FC \geq 2$, $p < 0.05$) in the expression of DH5α-pUC19 and with no changes ($-2 \leq Log_2FC \leq 2$) login the expression of DH5α-pUC19-*tmexCD1-toprJ1* were selected and sorted them by the absolute value of increasing or decreasing mRNA expression. RT-qPCR was used to further screen and validate the transcriptome-based *tmexCD1-toprJ1*-related biomarkers in clinical isolates. Firstly,

160 synonymous genes in *K. pneumoniae* according to the transcriptome initial screening were selected as the candidates (Table S4). Three *tmexCD1-toprJ1*-negative and three *tmexCD1-toprJ1*-positive *K. pneumoniae* isolates with clear backgrounds were selected and cultured at a concentration of 2 μg/mL for 60 min. *K. pneumoniae* 16S rRNA was used as an internal reference, and the value of Log_2FC was used to express the change of mRNA expression to verify the change of mRNA expression of *tmexCD1-toprJ1*-related biomarkers in the initial screening. $Log_2FC > 2$ or < -2 is the baseline from which mRNA expression changes significantly. A total of 12 genes were screened (Table S5). After being stimulated by tigecycline, there are eight significantly upregulated genes (*KPHS_33510*, *KPHS_47130*, *KPHS_02000*, *KPHS_33750*, *KPHS_25860*, *KPHS_09170*, *KPHS_11650*, *KPHS_33360*) and four significantly downregulated genes (*KPHS_50580*, *KPHS_20750*, *KPHS_31180*, *KPHS_50570*) in tigecycline-susceptible strains, while their mRNA expression did not change significantly in three *tmexCD1-toprJ1*-mediated tigecycline-resistant *K. pneumoniae* (Fig. 3 and S1). To verify the robustness of the assay developed, we matched the sequences of these 12 selected genes with 48,846 *K. pneumoniae* genomes available in the NCBI database to confirm their ubiquity. The results indicated that these twelve genes are relatively conserved in *K. pneumoniae* (Fig. S2).

Effect of tigecycline concentration changes on selected RNA biomarkers

In order to verify the relationship between tigecycline-stimulated concentration and the expression of 12 mRNA biomarkers screened, the changes of different mRNA expression levels were verified after 60 min of tigecycline stimulation at different concentrations. A tigecycline-susceptible *K. pneumoniae* and a *tmexCD1-toprJ1*-mediated tigecycline-resistant *K. pneumoniae* were stimulated with tigecycline from 0.03125 to 64 μg/mL. One hour after tigecycline stimulation, gene expression changes in 12 mRNA biomarkers of both strains were detected with RT-qPCR analysis. Of the 12 candidate genes, mRNA expression in four genes (*KPHS_02000*, *KPHS_11650*, *KPHS_33360*, *KPHS_20750*) was the most sensitive to stimulation at different concentrations of tigecycline (Fig. S3). From the results of column S in Fig. 4A to D, when the stimulus concentration of tigecycline exceeds 0.25 μg/mL, the mRNA expression of these four genes of tigecycline-susceptible *K. pneumoniae* began to change, and when the stimulus concentration exceeded 2 μg/mL, the change in mRNA expression reached a high level. However, under the stimulation of the same concentration of tigecycline, the mRNA expression of *tmexCD1-toprJ1*-mediated tigecycline-resistant *K. pneumoniae* did not change. Column R indicated that only when the tigecycline stimulation concentration exceeded 16 μg/mL did the mRNA expression of these four genes of *tmexCD1-toprJ1*-positive *K. pneumoniae* begin to show significant changes, but the specific values were still lower than tigecycline-susceptible *K. pneumoniae*.

Effect of tigecycline stimulation time changes on selected RNA biomarkers

The relationship between tigecycline stimulation time and the expression of the screened 12 mRNA biomarkers was also detected. After stimulation of 2 μg/mL tigecycline, the changes in different mRNA expression levels were verified

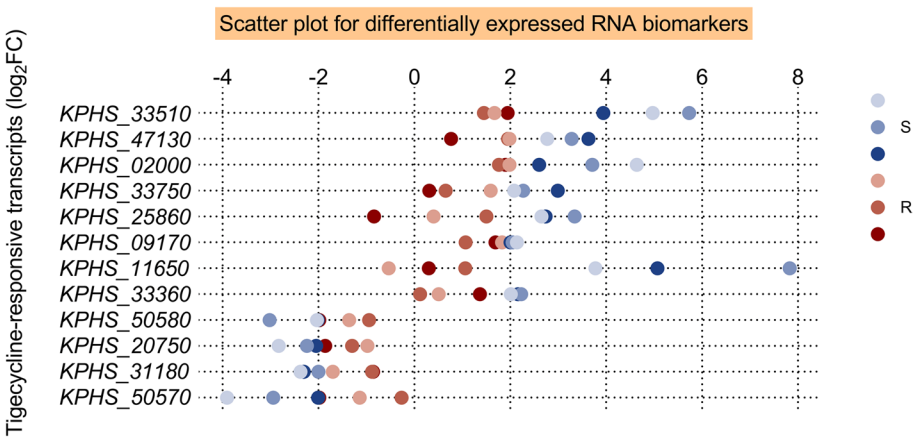

Fig. 3 RBAST distinguishes *tmexCD1-toprJ1*-negative and -positive isolates. Scatter diagram of 12 differentially RNA biomarkers across three *tmexCD1-toprJ1*-negative and three *tmexCD1-toprJ1*-mediated tigecycline-resistant *K. pneumoniae* after tigecycline exposure compared with their own control. The *tmexCD-toprJ*-negative *K. pneumoniae* were indicated in blue and the *tmexCD1-toprJ1*-positive tigecycline-resistant *K. pneumoniae* were indicated in red. 16S rRNA was the reference gene

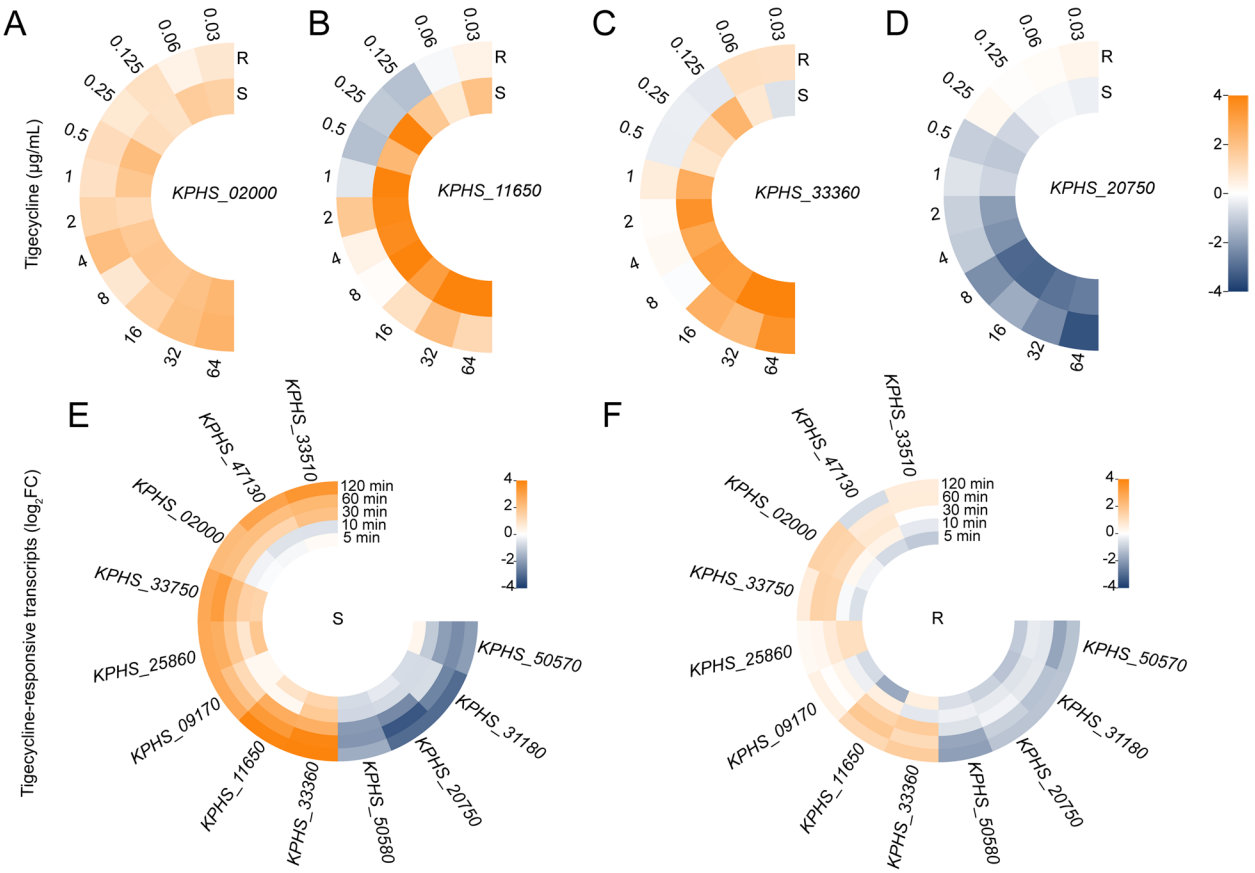

Fig. 4 Expression of selected RNA biomarkers upon different antibiotic exposure concentrations and times. Heatmaps of *KPHS_02000* (**A**), *KPHS_11650* (**B**), *KPHS_33360* (**C**), and *KPHS_20750* (**D**) biomarkers upon exposure to different concentrations of tigecycline. Heatmaps of 12 RNA biomarkers across exposure duration of tigecycline (**E** and **F**). The *tmexCD1-toprJ1*-negative isolate C11 was indicated as 'S', and the *tmexCD1-toprJ1*-positive isolate RGT40-1 was indicated as 'R'. 16S rRNA was the reference gene

after 5 to 120 min (Fig. S4). After tigecycline-susceptible *K. pneumoniae* was stimulated by tigecycline for 30 min, the expression of 12 related mRNA biomarkers changed to varying degrees. After 60 min of stimulation, the mRNA expression of all 12 selected genes changed significantly and remained until 120 min after stimulation (Fig. 4E). However, the mRNA expression of 12 genes in *tmexCD1-toprJ1*-mediated tigecycline-resistant *K. pneumoniae* did not significantly change within 120 min of stimulation (Fig. 4F). The results of the stimulation at different times and different concentrations once again demonstrate the feasibility of using changes in mRNA biomarkers to detect whether *K. pneumoniae* carries *tmexCD1-toprJ1* efflux pump gene clusters.

Shifts in 12 mRNA biomarkers of *Klebsiella pneumoniae* carrying the *tmexCD1-toprJ1* variant after tigecycline stimulation

With the widespread use of tigecycline, more and more *tmexCD-toprJ* variants have been discovered. In order to verify whether the developed RBAST for *tmexCD1-toprJ1*-positive *K. pneumoniae* can be similarly applied to detect *K. pneumoniae* mediated by other variants, different variant-mediated tigecycline-resistant engineered strains were constructed. Specific primers were designed to amplify the coding sequence of the *tmexCD-toprJ* resistance genes with their respective promoter fraction upstream of the coding sequence using high-fidelity enzymes, and the purified PCR gene products were linked to the linearized plasmid pUC19 by homologous recombination. MIC results showed that the constructed *tmexCD1-toprJ1*, *tmexCD2-toprJ2* and *tmexCD3-toprJ3*-positive *K. pneumoniae* exhibited tigecycline-resistant phenotype (MIC ≥ 8 μg/mL). After 60 min of tigecycline stimulation, the changes in mRNA expression of 12 selected genes were determined in different *tmexCD-toprJ* variants-mediated tigecycline-resistant *K. pneumoniae* (Fig. S5). As shown in Fig. 5, the expression of 12 mRNA biomarkers did not change significantly after stimulation in *K. pneumoniae* mediated by three different

tmexCD1-toprJ1 variants, indicating that this method is also stable and feasible to identify other *tmexCD-toprJ* variants-conferred *K. pneumoniae*.

Accuracy of *tmexCD-toprJ*-associated RBAST in *K. pneumoniae* isolates

To further evaluate the sensitivity and accuracy of *tmexCD1-toprJ1*-mediated tigecycline-resistant *K. pneumoniae* detection methods based on specific mRNA biomarkers, 38 isolates of *K. pneumoniae* were selected. After stimulation with 2 μg/mL tigecycline on these isolates, total RNA was extracted, reverse-transcribed and then subjected to RT-qPCR verification, and the sensitivity of 38 isolates to tigecycline was identified according to the results of changes in mRNA expression after tigecycline stimulation, and compared with the tigecycline sensitivity results obtained by broth microdilution method (Fig. 6 and S6). In order to make the results obtained in this experiment more accurate, in the analysis of the results of quantitative PCR, the mRNA expression of at least 10 genes after tigecycline stimulation was significantly upregulated or downregulated ($Log_2FC \geq 2$ or ≤ -2, $p < 0.05$) as the criterion for tigecycline-susceptible *K. pneumoniae* in the analysis of the results of RT-qPCR. In contrast, isolates of at least 10 of the 12 mRNA biomarkers whose mRNA expression did not change significantly after tigecycline stimulation ($-2 \leq Log_2FC \leq 2$, $p < 0.05$) were defined as *tmexCD-toprJ*-mediated tigecycline-resistant *K. pneumoniae*. Of the 38 isolated *K. pneumoniae* selected, 13 were defined as *tmexCD-toprJ*-negative tigecycline-sensitive *K. pneumoniae* (Fig. 6A) and 25 were defined as *tmexCD-toprJ*-positive tigecycline-resistant *K. pneumoniae* (Fig. 6B) based on specific mRNA biomarkers. After comparing the results of traditional broth microdilution method to identify bacterial sensitivity, the *tmexCD-toprJ*-positive detection method for *K. pneumoniae* based on specific mRNA biomarkers correctly distinguished 36 of the 38 isolated strains with 94% accuracy, including 13 of the total 15 tigecycline-sensitive *K. pneumoniae* and 23 of the total 23 *tmexCD-toprJ*-positive tigecycline-resistant *K. pneumoniae*. In addition, RBAST accurately classified *K. pneumoniae* isolates at a minimum of 10^6 CFUs (Fig. S7). The above results suggest that the AST based on specific mRNA biomarkers are effective in detecting *tmexCD-toprJ*-mediated tigecycline-resistant *K. pneumoniae*.

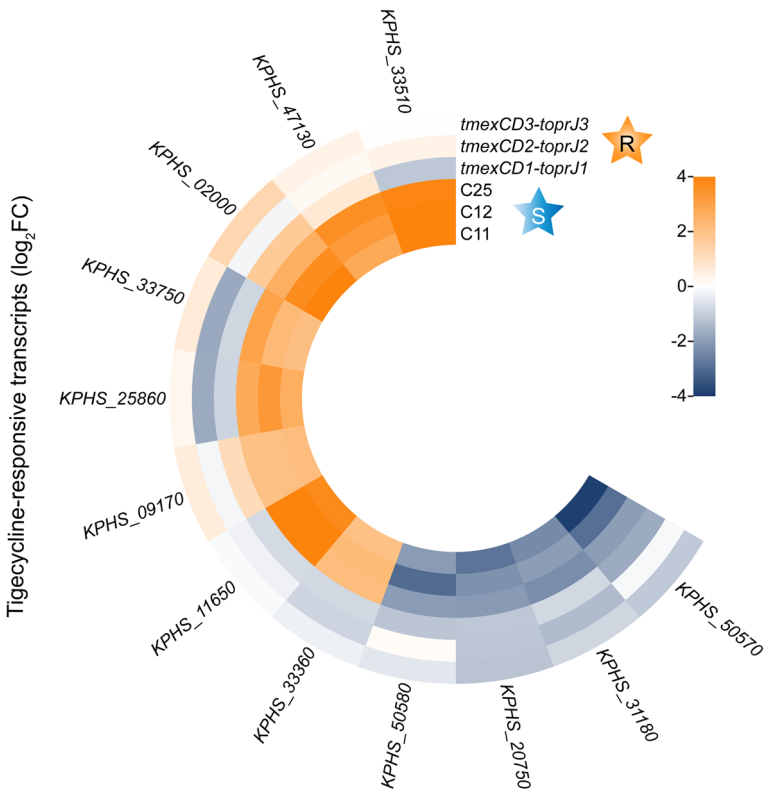

Fig. 5 RBAST detects different *tmexCD1-toprJ1* variants using the selected RNA biomarkers. Heatmap of 12 RNA biomarkers validation across *tmexCD-toprJ*-negative and different variants of *tmexCD-toprJ*-positive tigecycline-resistant after tigecycline exposure compared with their own control. The *tmexCD-toprJ*-negative *K. pneumoniae* were indicated as 'S', and the tigecycline-resistant *K. pneumoniae* carrying different *tmexCD-toprJ* variants were indicated as 'R'. 16S rRNA was applied as the reference gene

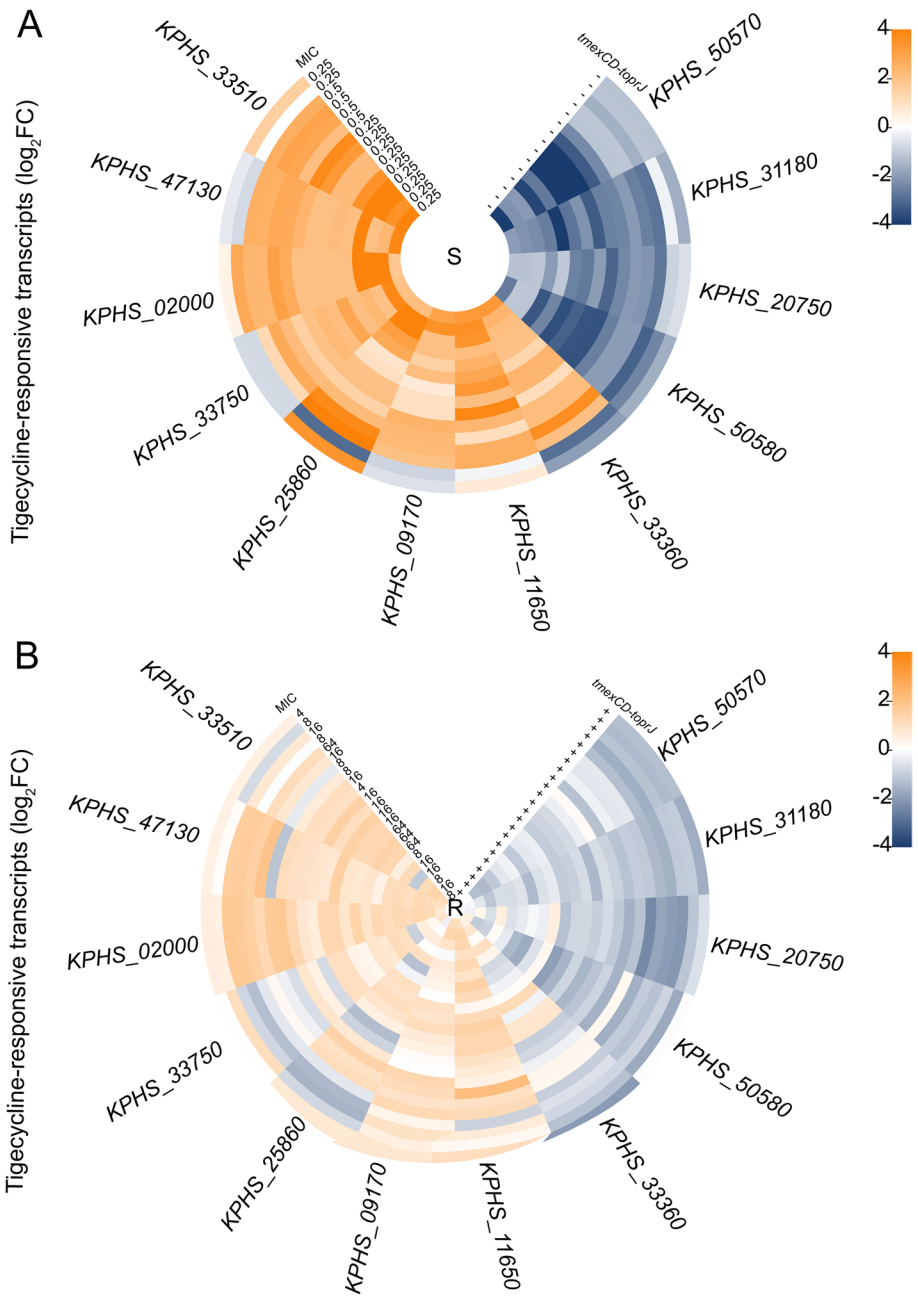

Fig. 6 RBAST accurately classifies *K. pneumoniae* isolates. Heatmap of changes in 12 validated RNA biomarkers across tigecycline-susceptible (**A**) and *tmexCD-toprJ*-positive tigecycline-resistant (**B**) *K. pneumoniae* after tigecycline exposure compared with their own control. Tigecycline-sensitive isolates were indicated as 'S', and *tmexCD-toprJ*-positive tigecycline-resistant isolates were indicated as 'R'. 16S rRNA was applied as the reference gene

Discussion

At present, a variety of reliable ASTs have been developed, but most require long enough time to support accurate results. The broth microdilution method is the gold standard for microbial susceptibility to tigecycline, and results are produced within 24 h of bacterial isolation and culture. Other commonly used tigecycline susceptibility detection methods include agar dilution method, disk-diffusion susceptibility testing, and gradient diffusion method. Although several assays for *tmexCD-toprJ*-mediated tigecycline-resistant bacteria have been reported recently, most of these methods are time-consuming and labor-intensive, and cannot detect

the genotype and phenotype of drug-resistant bacteria simultaneously [22, 23].

Instead of directly detecting cell viability, novel AST method indirectly determines microbial susceptibility by detecting other surrogate indicators of viability in microorganisms. Unlike DNA, mRNA acts as a bridge between DNA and proteins, and changes in levels can measure different stressors. Microorganisms sensitive to an antimicrobial drug experience significant changes in mRNA expression in some key genes after a short period of antibiotic exposure, but this change does not occur in drug-resistant bacteria, thus we can determine the susceptibility of these microorganisms to antimicrobials based on expression changes of specific genes after antibiotic exposure [25]. Until now, rapid RNA biomarker-based assays have been used to detect a wide range of bacterial susceptibility to antibiotics, including fluoroquinolones and colistin [26–31]. Likewise, RNA biomarkers have been applied to diagnose various disorders such as cancer, Parkinson's disease, and other infections [32–34].

In this study, we found that *tmexCD1-toprJ1*-negative and -positive bacteria exhibited different transcriptome changes after tigecycline exposure. In the *tmexCD1-toprJ1*-negative group, strong transcriptome changes occurred within minutes of antibiotic treatment, while fewer changes occurred in the *tmexCD1-toprJ1*-positive group. Based on these findings, we developed a rapid detection assay called RBAST to identify the tigecycline-susceptible *K. pneumoniae* using shifts in the expression of certain mRNA biomarkers after tigecycline stimulation. RBAST can complete the entire detection process in a total of 3 h from the exposure of tigecycline to the completion of the final RT-qPCR for a single sample, which was simple, fast and time-saving, and facilitates the clinical treatment of various infections caused by drug-resistant *K. pneumoniae*. Although RBAST has the advantages of high speed and accuracy, its efficiency cannot reach 100%, which may be related to the randomness of strains. Generally, some isolates have complex backgrounds, and when the strain is regulated by a variety of resistance genes, it may have an impact on RBAST, which needs further study.

Further, our study also provides deeper biological insights on the actions of tigecycline. In our 12 biomarkers, the gene expression of *KPHS_33510* that encodes manganese efflux protein and ribosome assembly factor *KPHS_47130* was upregulated after tigecycline stimulation. *KPHS_33510* is crucial to evading manganese toxicity in bacteria [35]. *KPHS_47130* can suppress protein synthesis of ribosome 30S subunits [36]. Meanwhile, the gene expression of *KPHS_02000* that regulates 50S ribosomal protein L7/L12 and *KPHS_33750* that regulates CopC domain-containing protein YobA was also upregulated. These results were consistent with the damage of ribosome function by tigecycline.

Conclusions

In conclusion, we successfully established a rapid and accurate RBAST assay based on changes in the transcription levels of *tmexCD1-toprJ1*-negative and -positive bacteria after tigecycline treatment. Candidate mRNA biomarkers can also be used for the detection of tigecycline-resistant *K. pneumoniae* mediated by other variants of *tmexCD-toprJ*. Compared with traditional antimicrobial susceptibility detection methods, the accuracy of this method is more than 90%.

Materials and methods

Bacterial strains

In this study, *E. coli* DH5α was used as a reference strain. All tested *K. pneumoniae* strains isolated from pork [37], poultry and human [38, 39], available as Supplementary Tables 1 and 2, were chosen and preserved in College of Veterinary Medicine, Yangzhou University, China. Drugs used in this study were purchased from Yuanye Bio-Technology (Shanghai, China).

Minimal inhibitory concentrations (MICs) and PCR determination

E. coli ATCC 25922 was used as a control by broth microdilution according to EUCAST clinical breakpoints methods. In brief, 10^6 CFU per mL bacteria were co-cultured with two-fold dilutions of tigecycline at concentrations ranging from 0.25 to 128 μg/mL in 96-well plates for 18 h at 37 °C in the dark. Tests were performed in three biological replicates containing negative and positive controls. The MIC value represents the lowest concentration of tigecycline at which bacterial growth is inhibited. Strains with MIC values > 0.5 μg/mL were identified as tigecycline resistant. PCR primers were used to screen for *tmexCD-toprJ* genes in all *K. pneumoniae* isolates as previously described. Subsequently, the PCR products were purified and Sanger sequencing was performed to confirm gene identity.

Plasmids and strains construction

The standard *tmexCD1-toprJ1*, *tmexCD2-toprJ2*, *tmexCD3-toprJ3* gene, and their respective promoters were amplified by PCR analysis. Primers and templates for amplification were shown in Supplementary Table 3. The purified nucleic acid was cloned into a pUC19 vector. The constructed recombinant expression vector was transformed into DH5α competent cells. Transformant screens were made using Luria Broth (LB) agar plates containing ampicillin (100 μg/mL) and tigecycline (2 μg/mL). The positive clones were confirmed by Sanger

sequencing. The empty vector pUC19 was converted into *E. coli* DH5α as a control. Plasmid Mini Kit I (Omega, Norcross, USA) was used to extract plasmids from *E. coli* DH5α and convert them into *K. pneumoniae* ATCC 700603 competent states.

RNA extraction and sequencing after antibiotic exposure

Single colony of DH5α-pUC19-*tmexCD1-toprJ1* and DH5α-pUC19 were cultured overnight at 37 ℃ in LB solution supplemented with ampicillin (100 μg/mL) to OD_{600} of 1.0. A range of 20 to 60 min is the doubling time of *K. pneumoniae*. Tigecycline treatment was set to 60 min to ensure that the exposure was long enough to produce significant changes in the transcriptome profile of *K. pneumoniae*. The treatment group was exposed to tigecycline at a concentration of 2 μg/mL for 60 min at 37 °C, whereas the control group was not subjected to any treatment.

RT-qPCR analysis

Bacterial RNA was extracted with the TRIzol reagent, then the HiScript®III RT SuperMix for qPCR (+GDNA-WIper) Kit (Vazyme Biotech, China) was used to reverse it into cDNA. RT-qPCR analysis was performed by using the optimized primers as shown in Table S5 and the ChamQ™SYBR® Color qPCR Master mix Kit (Vazyme Biotech, China). Experiments were conducted with three biological replicates. Comparative CT method was applied to calculate the relative expression levels of representative genes [40]. 16S rRNA was employed as a reference gene.

RNA sequencing and data preprocessing

TRIzol® Reagent was used to extract total RNA from the cells (InvitroGen, Carlsbad, CA) and DNase I (TaKara, Shiga, Japan) was used to remove the genomic DNA. Then 2100 Bioanalyser (Agilent, Palo Alto, CA) was used to determine the RNA quality and ND-2000 (NanoDrop, Wilmington, DE) was used to quantify. 2 μg of total RNA was used to prepare RNA-seq transcriptome library by TruSeq™ RNA sample preparation kit from Illumina (San Diego, CA). Illumina HiSeq×10 (2×150 bp read length) was used to sequence the library. DESeq2 [41] was used to identify DEGs. Principal component analysis (PCA) was performed using FactoMineR and plotted using ggplot2.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s44280-023-00025-9.

Additional file 1: Supplementary Fig. 1. RBAST distinguishes tigecycline-susceptible and *tmexCD1-toprJ1*-medicated tigecycline-resistant isolates. **Supplementary Fig. 2.** Percentage of the selected biomarker genes in *K. pneumoniae*. **Supplementary Fig. 3.** Expression of candidate mRNA biomarkers upon different tigecycline exposure concentrations. **Supplementary Fig. 4.** Expression of candidate mRNA biomarkers upon different tigecycline exposure times. **Supplementary Fig. 5.** RBAST detects different *tmexCD-toprJ* variants using the selected RNA biomarkers. **Supplementary Fig. 6.** RBAST accurately classifies *K. pneumoniae* isolates. **Supplementary Fig. 7.** RBAST accurately classifies *K. pneumoniae* isolates at a minimum of 10^6 CFUs. **Supplementary Table 1.** Antimicrobial susceptibility profile of tested isolates of *tmexCD-toprJ*-positive *K. pneumoniae* (MIC, μg/mL). **Supplementary Table 2.** Antimicrobial susceptibility profile of tested isolates of *tmexCD-toprJ*-negative *K. pneumoniae* (MIC, μg/mL). **Supplementary Table 3.** Primer sequences used for *tmexCD-toprJ* variant constructions. **Supplementary Table 4.** List of differentially expressed RNA biomarkers in tigecycline-susceptible and *tmexCD1-toprJ1*-mediated tigecycline-resistant *E. coli* compared to their own control. **Supplementary Table 5.** Primer sequences used for validation of 12 selected RNA markers using RT-qPCR analysis.

Authors' contributions

Y.L. and Z.W. designed and conceived the project; F.Y., H.Z. and S.Z. performed all experiments; F.Y. analyzed the data and prepared all figures; Y.L. and F.Y. wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

RNA-sequencing data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database (PRJNA948819).

The genome sequences in this study have been deposited in NCBI database under the accession numbers (PRJNA904099), or figshare database reference: (https://doi.org/10.6084/m9.figshare.23713818), (https://doi.org/10.6084/m9.figshare.23715633).

Declarations

Competing interests

The authors declare that they have no competing interests.

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