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Cas14VIDet: A visual instant method free from PAM restriction for antibiotic resistance bacteria detection

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ABSTRACT

A personalized treatment strategy that selects sensitive antibiotics based on *Helicobacter pylori* (*H. pylori*) resistance genes is currently the most effective approach to address the challenge of *H. pylori* eradication. However, the widespread adoption of this strategy is hindered by the long processing times and high costs associated with traditional resistance gene detection methods. In this study, we combined ultra-fast PCR with CRISPR/Cas14 into a single reaction system, establishing a rapid, one-pot visual platform named Cas14VIDet (Cas14-based Visual Instant Detection) for detecting *H. pylori* resistance genes. Cas14VIDet does not require a PAM sequence and excels in identifying single nucleotide polymorphisms, with the detection sensitivity approaching the level of a single bacterial colony (10^0 CFU/mL). The entire detection process can be completed within 10 min, and results are directly observable with the naked eye. We validated Cas14VIDet by testing 50 clinical samples and compared it with Sanger sequencing. The results showed that Cas14VIDet achieved 100% sensitivity, 100% specificity, and 100% accuracy in detecting *H. pylori* resistance genes to levofloxacin. Therefore, we believe this method holds great potential for rapid detection of *H. pylori* resistance, potentially supporting personalized treatment of *H. pylori* infections in the future.

1. Introduction

Helicobacter pylori (*H. pylori*) is a Gram-negative bacterium that infects nearly half of the global population (Zhou et al., 2023; Ding et al., 2022; Lai et al., 2022). Treating *H. pylori* infection is considered the most critical modifiable factor in the treatment and prevention of gastric diseases, including gastric cancer (Du et al., 2020; Ding et al., 2022; Sugano et al., 2015). However, despite decades of advancements and numerous consensus meetings offering the latest treatment recommendations, the overall success rate of *H. pylori* treatment remains low compared to other infectious diseases (Graham et al., 2022). The significant strain variation of *H. pylori* species among individuals, along with the increasing antibiotic resistance, are the primary reasons for the difficulty in improving the eradication success rate of *H. pylori* (Tshibangu-Kabamba and Yamaoka, 2021). The passive use of empirical treatment regimens, due to the difficulty in accurately selecting antibiotics sensitive to *H. pylori*, not only increases the bacterium's resistance but also adds to the patient's antibiotic burden (Graham, 2024; Graham and Liou, 2022). Therefore, screening for *H. pylori* antibiotic susceptibility before treatment would be an effective approach to overcoming the current challenges in *H. pylori* therapy.

Currently, clinical methods for detecting *H. pylori* resistance can be categorized into antibiotic susceptibility testing and resistance gene detection assays (Pohl et al., 2019). Antibiotic susceptibility testing

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evaluates bacterial sensitivity by determining the antibiotic concentration that inhibits or kills the bacteria, which is the most common method used in hospitals for *H. pylori* resistance detection. The widely used methods include the disk diffusion method, E-test, and agar dilution method. The disk diffusion method and E-test are simple to perform and cost-effective, but they have lower accuracy and are time-consuming, often requiring over 10 days from sample collection to bacterial isolation and culture (Shakir et al., 2023). This lengthy process increases the risk of contamination by other bacteria during culturing. The agar dilution method is more accurate but requires advanced technical expertise and is more expensive. Additionally, the *H. pylori* culture process using the agar dilution method is also time-intensive. (Shakir et al., 2023). In contrast, *H. pylori* resistance gene detection does not require bacterial culture and can rapidly and efficiently assess antibiotic resistance by detecting resistance genes directly from gastric mucosal tissue samples (Li et al., 2022). Common methods include polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), fluorescence in situ hybridization (FISH), and real-time quantitative PCR (RT-PCR). However, these techniques require sophisticated instrumentation and skilled personnel, significantly limiting their utility in point-of-care testing (POCT) or low-resource settings (Liu et al., 2024). Therefore, there remains an urgent need to develop a novel *H. pylori* resistance gene detection method that offers high specificity, sensitivity, and cost-effectiveness while being rapid, portable, and easy to use.

Recently, POCT based on Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated (Cas) proteins has shown great potential for nucleic acid detection with enhanced robustness, speed, low cost, specificity, and sensitivity, fulfilling unmet needs (Wang et al., 2023). In previous research, our team developed several POCT platforms based on the CRISPR system, enabling rapid detection of acute respiratory syndrome coronavirus 2 (SARS-cov-2), *Escherichia coli*, *Vibrio parahaemolyticus*, and Mycoplasma (Wang et al., 2021; Wang et al., 2021; Wang et al. 2017, 2019, 2022). Guided by CRISPR-derived RNA (crRNA) complementary to the target sequence, and located by recognizing the protospacer adjacent motif (PAM), the CRISPR/Cas complex could specifically recognize and cleave target nucleic acids. When combined with fluorescent reporter, the CRISPR/Cas complex would trans cleave reporter and generate detectable signal, thus achieve POCT detection. However, CRISPR detection systems were restricted by PAM sequence, which greatly limited their application scope. Therefore, build a CRISPR system free from PAM sequence and owns single nucleotide polymorphism (SNP) detection specificity is imperative for drug-resistant bacteria detection (Harrington et al., 2018; Karvelis et al., 2020).

Here, we developed a visual instant system based on Cas14 for detecting the levofloxacin resistance gene in *H. pylori* named High Sensitivity Cas14 based Visual Instant Detection (Cas14VIDet). Our results demonstrate that this detection system can accurately identify mutation sites in the levofloxacin resistance gene (*GyrA*) of *H. pylori* and directly display the results via fluorescence, exhibiting high sensitivity and specificity in clinical samples (Fig. 1). We believe this detection system could facilitate the rapid identification of *H. pylori* resistance genes, thereby aiding in personalized treatment and significantly improving the eradication success rate of *H. pylori*.

2. Methods

2.1. Materials

Un1Cas12f1 (Cas14a) Nuclease (including Holmes buffer) (32119) were obtained from Tolo Biotech. Co., Ltd (Shanghai, China). All primers, guide RNA (gRNA), and ssDNA (quenched fluorescent DNA reporter FAM-TTATT-BHQ$_1$) were synthesized by Sangon Biotech. (Shanghai, China). HiScribe T7 High YieldRNA Synthesis Kit (E2040S) were purchased from New England Biolabs (Ipswich, MA, U.K.). Taq Hot Start Version (R007) was obtained from Takara Bio Inc. (Dalian, China). HiPure RNA Pure Micro kit (R4122-02) was purchased from Magen Biotech. (Guangzhou, China). All clinical samples were provided by the Department of Gastroenterology, the First Affiliated Hospital of Naval Medical University, and the study was approved by the Ethics Committee of the First Affiliated Hospital of Naval Medical University (CHEC2021-131), with verbal notification of obtaining consent from all patients involved.

2.2. Patient samples and H. pylori culture

The gastric tissue obtained via endoscopy was divided into two parts. One part was used for immunohistochemical staining to microscopically determine the presence of *H. pylori* infection in the gastric tissue. The other part was uniformly smeared on blood agar plates containing 5% sheep blood and 1% mixed antibiotics (10 μg/mL vancomycin, 5 μg/mL trimethoprim lactate, 5 μg/mL cefsulodin sodium, and 5 μg/mL polymyxin B sulfate). *H. pylori* strains were then selected and cultured for further expansion in a microaerobic environment (37 °C, 10% CO_2, 5% O_2). For genomic DNA extraction, resuspend the cultured *H. pylori* cells in solution. Then, place the suspension in a water bath at 99 °C for 3 min to obtain *H. pylori* DNA using the heat lysis method.

2.3. Design and synthesis of gRNA

The gRNA was designed using SnapGene software version 7.2 (https://www.snapgene.com/). Firstly, we amplify the template using PCR. Briefly, the reaction mixture contains 1.25 U TaKaRa Taq HS, 5 μL 10 × PCR buffer (including 10 mM Tris HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 4 μL dNTP (each), 2 μL forward primer (10 μM), 2 μL reverse primer (10 μM), and 34.75 μL sterile water. PCR was carried out with 5 min hot start at 95 °C, followed by 40 cycles of [95 °C for 20 s, and 55 °C for 30 s, and 72 °C for 30 s]. A final extension was performed at 72 °C for 5 min, and the reaction was then held at 4 °C for storage. The gRNA was transcribed *in vitro* using the HiScribe™ T7 High Yield RNA Synthesis Kit (Supplement Fig. 1). Briefly, the reaction mixture (30 μL) contained 5 μL PCR amplicon as template, 10 μL NTP mix, 2 μL T7 RNA polymerase, and 13 μL nuclease-free water. The reaction was incubated at 37 °C for 24 h.

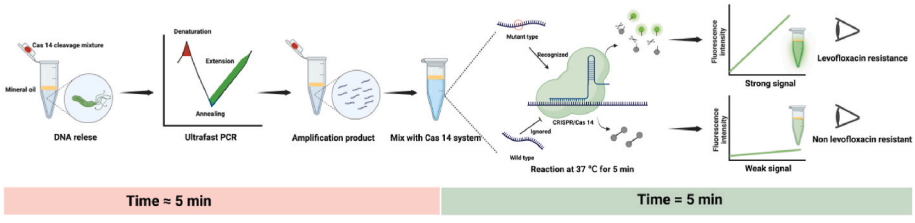


Fig. 1. Scheme of Cas14VIDet for ultrafast and visual drug-resistance *H. pylori* detection. A one-pot method based on "CRISPCR/Cas14" was proposed for ultrafast and visual detection of *H. pylori* levofloxacin resistance genes. After isolating *H. pylori* from gastric tissue, asymmetric ultrafast PCR amplification was used to obtain single-stranded DNA of the target gene. Subsequently, the Cas14 cleavage system was mixed with the ultrafast PCR solution, incubated at 37 °C for 5 min, and the fluorescence result was visually read under blue light.

Subsequently, 20 U RNase inhibitor, 5 μL DNase I, 5 μL DNase I buffer, and 5 μL nuclease-free water were added to make a final reaction volume of 50 μL, and the mixture was incubated at 37 °C for 1 h. The gRNA was then purified using the HiPure RNA Pure Micro Kit according to the manufacturer's instructions. All DNA sequences used in this experiment were listed in Supplement materials.

2.4. Ultrafast PCR amplification

Primers for PCR amplification was designed with SnapGene software as described above. The ultrafast PCR amplification system (50 μL) contains 12.5 U TaKaRa Taq HS, 5 μL 10 × PCR buffer (including 10 mM Tris HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 4 μL dNTP (each), 2 μL forward primer (1 μM), 2 μL reverse primer (10 μM), and 33.5 μL sterile water. Ultrafast PCR was carried out with 30 s hot start at 98 °C, followed by 40 cycles of [98 °C for (1 s or 2 s or 5 s or 10 s) and 55 °C for (2 s or 3 s or 5 s or 10 s)], respectively. The amplified products were analyzed with real-time fluorescence amplification curve and electrophoresed at 2 % agarose gel under 120 V for 40 min.

2.5. One-pot visual ultrafast PCR-CRISPR detection

To detect Cas14VIDet, 10 μL of ultrafast PCR reaction mixture (2.5 U TaKaRa Taq HS, 1 μL 10 × PCR buffer, 0.8 μL dNTP (each), 0.4 μL forward primer (1 μM), 0.4 μL reverse primer (10 μM), and 6.7 μL sterile water), including 1 μL of clinical sample DNA, was added to the bottom of a tube, followed by covering it with 20 μL of mineral oil. The Cas14 cleavage system was added to the tube cap. The cleavage system was prepared by mixing 500 nM gRNA, 1 μM reporter, 2 μL Holmes buffer, 0.25 μM Cas14, 4 U of RNase inhibitor, 7 mM magnesium acetate, and 3 μL of RNase-free sterile water. The oil layer physically separated the Cas14 cleavage system from the amplification system at the bottom of the tube to prevent inactivation of Cas14 by high temperature. When ultrafast PCR was completed, the Cas14 cleavage system was mixed with the ultrafast PCR product by shaking. The entire system was incubated at 37 °C for 5 min. Finally, all reaction tubes were observed under blue light, and images were captured using a smartphone.

2.6. Statistical analysis

All data were expressed as the mean ± standard deviation and analyzed with R statistical software 4.4.0 (www.r-project.org) and GraphPad Prism v. 9.5 (GraphPad Software, La Jolla, CA, USA). All bar charts, line graphs, AUC curves, and Venn diagrams were generated using GraphPad. The disparity between the two groups was evaluated utilizing the student's t-test, whereas the variances among three or more groups were assessed via ANOVA. P value < 0.05 was considered statistically significant.

3. Results

3.1. Single-base recognition capability of CRISPR/Cas14

In this research, we evaluated the mismatch tolerance of Cas14 by

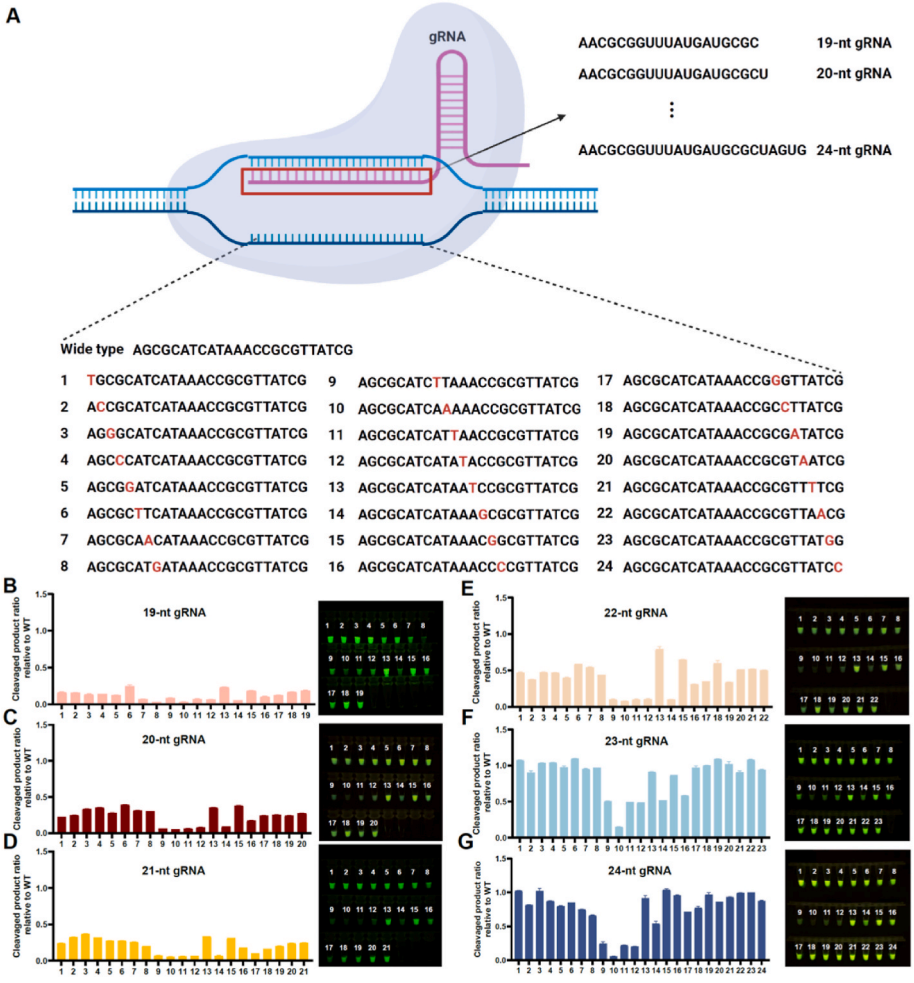


Fig. 2. Investigation of CRISPR/Cas14's single-nucleotide recognition capability. (A) Schematic diagram of mismatch bases. Single base recognition ability with gRNA lengths of 19 (B), 20 (C), 21 (D), 22 (E), 23 (F), and 24 (G) bases. Data are presented as the mean ± SD (n = 3).

introducing single nucleotide mismatches at target sites within various single-stranded DNA (ssDNA) substrates using gRNAs of different lengths (Fig. 2A). Specifically, gRNAs ranging from 19 to 24 nucleotides (nt) in spacer length were combined with the Cas14 reaction system, a self-quenching probe, and 1 μL of ssDNA template. This mixture was incubated at 37 °C for 30 min. Subsequently, fluorescence intensity was measured under blue light, and specific fluorescence values were recorded using a quantitative PCR instrument.

Analysis of the fluorescence intensity revealed significant specificity at position 10 across all gRNA lengths tested. Further analysis showed that 19-nt gRNAs were intolerant of single mismatches at positions 7–12 and 14 (Fig. 2B). The gRNAs of 20-nt, 21-nt, and 22-nt lengths showed intolerance to single mismatches at positions 9–12 and 14 (Fig. 2C–E). The gRNAs of 23-nt and 24-nt lengths were intolerant of single mismatches exclusively at position 10 (Fig. 2F and G). A comparative analysis of gRNA performance at position 10 indicated that the 24-nt gRNA had a fluorescence ratio of 0.052 relative to the wild type (WT), which was significantly lower than that of 21-nt (0.054), 22-nt (0.078), and 23-nt (0.147) gRNAs. Moreover, the 24-nt gRNA demonstrated superior CRISPR cleavage efficiency, compared to the 19-nt and 20-nt gRNAs (Supplement Fig. 2). Therefore, considering both gRNA specificity and work efficiency, the 24-nt gRNA was selected for the development of the Cas14VIDet method.

3.2. Ultrafast Cas14VIDet system built up

To minimize the detection time, we built ultrafast PCR system to achieve effective amplification (Fig. 3A). Using 5 × 10^2 copies of *H. pylori* (wild-type) DNA as the template, we adjusted the denaturation and annealing time of ultrafast PCR and compared the results with standard PCR. Gel electrophoresis showed amplicon band of 300 bp at target position in both standard and ultrafast PCR system, demonstrating specific amplification of ultrafast PCR (Fig. 3B). We further optimized the ultrafast PCR reaction time by adjusting the denaturation and annealing times. The results indicated that setting the denaturation time to 2 s and the annealing time to 3 s for 40 cycles provided the best amplification effect while shortening the time. Additionally, we compared the ultrafast PCR system we developed with standard PCR in

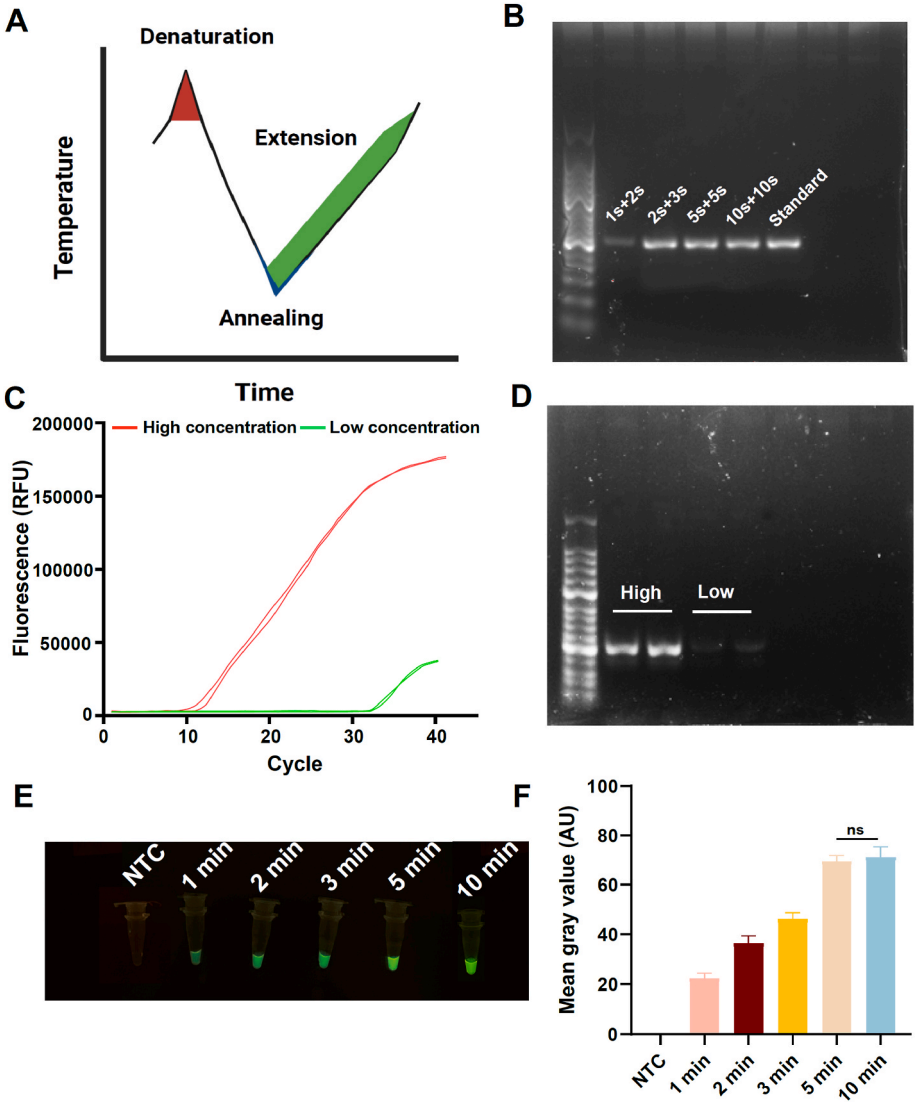


Fig. 3. Feasibility investigation of Cas14VIDet system. (A) Schematic diagram of ultrafast PCR. (B) Confirmation of optimal annealing and extension times in the ultrafast PCR protocol. (C) Amplification curve of high concentration (10^6 CFU/mL) and low concentration (10^0 CFU/mL) samples amplified by standard PCR. (D) Ultrafast PCR amplification of gel plots for high concentration and low concentration samples. (E) Time range evaluation of the CRISPR/Cas14 cleavage system, from 1 min to 10 min. (F) Quantitative fluorescence analysis of the CRISPR/Cas14 cleavage system at different reaction times. Data are presented as the mean ± SD (n = 3), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

amplifying high-concentration (10^6 CFU/mL) and low-concentration (10^0 CFU/mL) samples. The results indicated that the amplification effectiveness of ultrafast PCR is comparable to that of standard PCR (Fig. 3C and D). Consequently, we established an ultrafast PCR system with an initial heat activation at 95 °C for 30 s, followed by 40 cycles of 95 °C denaturation for 2 s and 60 °C annealing/extension for 3 s. This ultrafast PCR system was used in subsequent assays.

Then, we developed a CRISPR/Cas14 cleavage system based on Cas14, gRNA, reporter, and 10 μL of amplicon. By optimizing the cleavage time, we identified an optimal CRISPR/Cas14 cutting system. The results showed that fluorescence intensity increased over time and became visibly detectable to the naked eye after 5 min of incubation at 37 °C (Fig. 3E). There was no significant difference in fluorescence intensity between 5 min and 10 min incubation time (Fig. 3F). Therefore, for subsequent assays, we set the CRISPR/Cas14 detection system reaction time to 5 min.

3.3. Ultrafast Cas14VIDet system for H. pylori drug-resistance gene detection

H. pylori is a rod-shaped Gram-negative bacterium, characterized by the presence of 5–7 flagella, and mutations in its GyrA gene can confer resistance to levofloxacin (Fig. 4A). Mutations in the *GyrA* are commonly found at amino acid 87 and 91 within the quinolone resistance-determining region. Using the *GyrA* of *H. pylori* as an example, we explored the capability of the Cas14VIDet system in identifying resistance gene mutations (Fig. 4B). Based on the common mutation types at these two sites, we designed seven different gRNAs. Subsequently, using the Cas14VIDet system, we tested target with corresponding base mutations and wild-type *H. pylori* without mutations using the designed gRNAs. Results showed that all seven gRNAs performed well in detecting *H. pylori* strains with the corresponding base mutations, with fluorescence values significantly higher than those for wild-type strains (Fig. 4C). Then, we mixed the seven different gRNAs and conducted detection on samples with the seven distinct mutation sites individually. The mixed gRNA performed well, distinguishing between levofloxacin-resistant and wild-type *H. pylori* strains based on fluorescence intensity under blue light (Fig. 4C). Thus, we used this mixed gRNA approach for subsequent experiments.

To further verify the specificity of Cas14VIDet system, we tested different rod-shaped Gram-negative including *Vibrio parahaemolyticus*, *Salmonella*, *Klebsiella pneumoniae*, and *Escherichia coli*. Gel electrophoresis results showed that only the *H. pylori* resistant strains were amplified, while no amplification was observed for other bacteria (Fig. 4D). When detecting *H. pylori* samples, both resistant and wild-type strains were amplified (Fig. 4E). This indicates that Cas14VIDet system could specifically amplify *H. pylori* strains but can not amplify other bacteria or viruses. Additionally, for all detections, only levofloxacin-resistant samples produced bright fluorescence, while other samples did not, demonstrating high specificity of the proposed method (Fig. 4D and E).

3.4. Sensibility evaluation of Cas14VIDet for H. pylori drug-resistance gene detection

Then, we explored the sensitivity of Cas14VIDet system by testing seven 10-fold serial dilutions of the levofloxacin-resistant *H. pylori* strains. We first demonstrated the bacterial colony counts within the concentration range of 10^0–10^6 CFU/mL through plate spreading. From the plating results, it was evident that when the bacterial concentration was diluted to 10^0 CFU/mL, the colonies on the plate were very sparse (Fig. 5A). Using the constructed detection system, we tested the levofloxacin-resistant *H. pylori* strains with concentration ranging from 10^0 to 10^6 CFU/mL. The results indicate that although Cas14VIDet could achieve precise quantification of bacterial counts, we observed that the fluorescence intensity varied with changes in template concentration. This suggests that a semi-quantitative analysis of bacterial concentration could be achieved based on fluorescence intensity to some extent (Fig. 5B and Supplement Fig. 3). When template concentration was 10^6 CFU/mL, the fluorescence was the strongest inside tube; at a concentration of 10^0 CFU/mL, the fluorescence intensity was weak but was still distinguishable by the naked eye; when there was no template, absolutely no fluorescence generated inside the tube (Fig. 5B). This demonstrated that the Cas14VIDet system possesses high sensitivity, capable of clearly detecting bacterial concentrations at nearly single colony level. Additionally, we also used PCR to detect *H. pylori* at different concentrations. The results show that qRT-PCR could detect bacteria at a concentration as low as 10^0 CFU/mL (Supplement Fig. 4), which is consistent with the sensitivity of the Cas14VIDet system we developed. Furthermore, we plotted the linear relationship between the Ct value of PCR and the endpoint fluorescence of Cas14VIDet, with an R^2 value of 0.9898, indicating a strong linear correlation (Supplement Fig. 5). This demonstrates that the detection accuracy of the Cas14VIDet method is equivalent to that of the PCR method, further confirming the high sensitivity of Cas14VIDet for pathogen detection.

3.5. Application of Cas14VIDet for H. pylori drug-resistance gene detection

To investigate the diagnostic reliability of Cas14VIDet, we detect 50 clinical samples infected with *H. pylori* which confirmed by immunohistochemistry. Briefly, we cultured *H. pylori* strains isolated from gastric tissues of patients and further tested their levofloxacin resistance with Cas14VIDet (Supplement Fig. 6). The presence of resistance gene mutations had been previously confirmed by sequencing, identifying 35 levofloxacin-resistant and 15 non-resistant strains. In this study, sequencing results served as the standard reference.

Using the Cas14VIDet system, we found that all strains identified as levofloxacin-resistant through sequencing were successfully detected by Cas14VIDet. Additionally, all non-resistant strains except one were accurately identified by Cas14VIDet as levofloxacin-susceptible (Fig. 6A and Supplement Fig. 7). The Venn diagram indicated that Cas14VIDet's capability to identify levofloxacin-resistant *H. pylori* strains was comparable to sequencing (Fig. 6B). Additionally, the system's performance was demonstrated by the receiver operating characteristic (ROC) curve (Fig. 6C), which showed an area under the curve (AUC) of 1.00 for detecting *GyrA* gene mutations in clinical samples, indicating high diagnostic capability. Furthermore, using sequencing as the standard for *H. pylori* resistance detection, the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of Cas14VIDet for detecting resistance genes in clinical samples were 100%, 100%, 100%, and 100%, respectively (Fig. 6D). Therefore, the proposed Cas14VIDet method demonstrated high specificity and sensitivity for clinical samples detection.

4. Disscussion

In this study, we developed a novel method for detecting antibiotic resistance genes in *H. pylori* based on CRISPR/Cas14 technology. This method enables rapid detection of *H. pylori* resistance genes with characteristics of simplicity, high sensitivity and specificity, low cost, and easy to implement. It holds promise for assisting doctors in effectively selecting antibiotics, thus achieving the goal of personalized treatment for *H. pylori* infections.

We first compared gRNAs ranging from 19 to 24 nucleotides in spacer length and found that all gRNA lengths exhibited significant specificity at the 10th position. Additionally, CRISPR cleavage efficiency increased with longer gRNAs. Considering both the specificity and efficiency of gRNAs, we selected the 24-nt spacer of gRNA for our subsequent experiments. We did not explore gRNAs longer than 24 nt, as our results showed that the 24-nt gRNA was sufficient for rapid and accurate detection of single-base mutations, while increasing the gRNA length

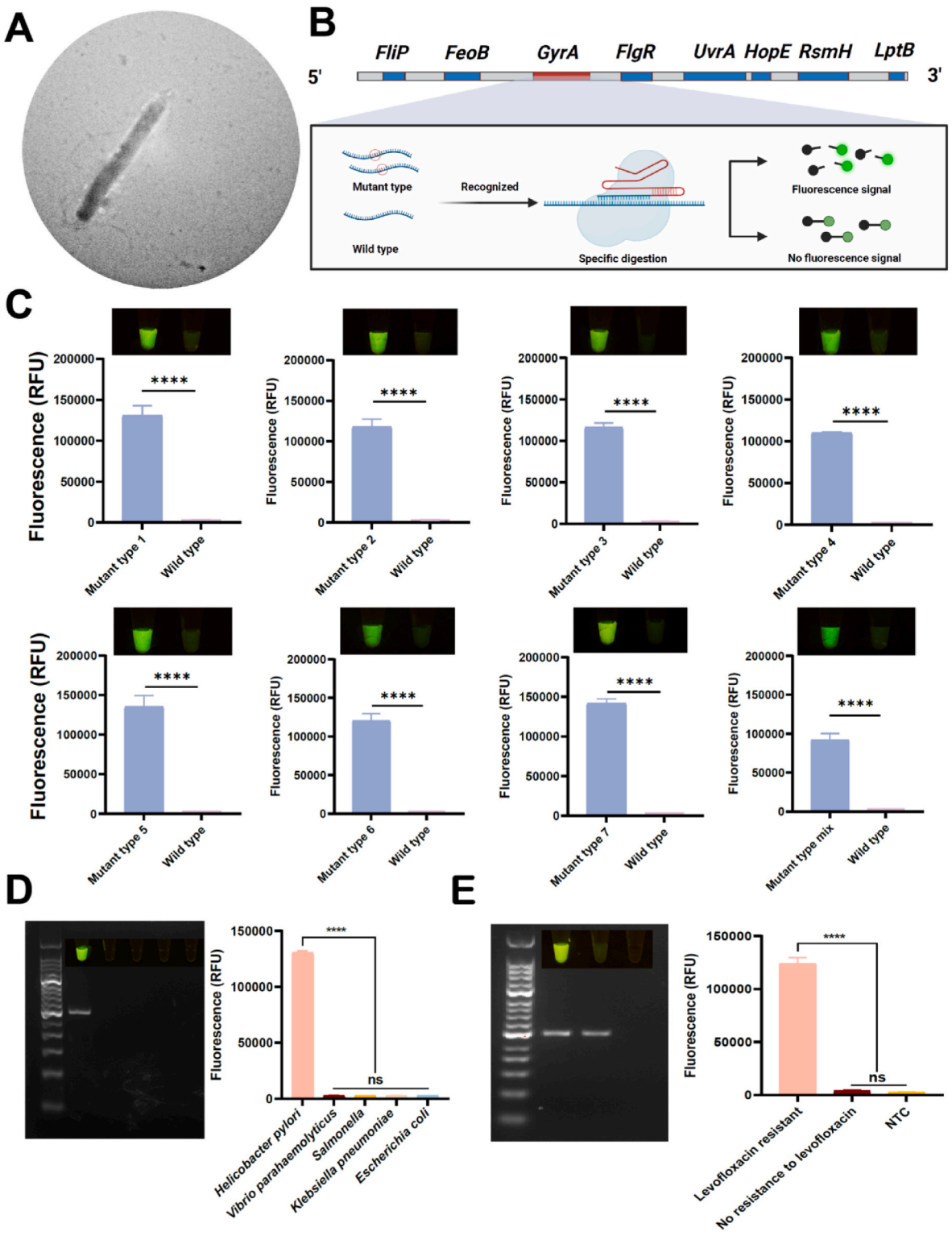


Fig. 4. Exploration of Cas14VIDet system in detecting antibiotic resistance genes of *H. pylori*. (A) TEM images of *H. pylori* in clinical samples. (B) Schematic diagram of CRISPR/Cas14 detection system recognizing *H. pylori* levofloxacin resistance gene *GyrA*. (C) Fluorescence bar and tube charts of detecting seven common mutation types in *H. pylori* levofloxacin-resistant strains with different gRNAs and mixed gRNAs. The target sequences and gRNAs for mutant types 1–7 and the wild-type can be found in the supplementary materials. The gRNA corresponding to the target of mutant type 1 is referred to as mutant type 1 gRNA, and so on for the others. The gRNA in the mutant type mix group was prepared by mixing the seven gRNAs in equal volume proportions. We used the mixed gRNA to detect the seven different targets individually, presenting the results in a bar graph. Additionally, a representative image of the fluorescent tubes is provided for visual reference. (D) Left: Gel images of ultrafast PCR amplification of *H. pylori*, *Vibrio parahaemolyticus*, *Salmonella*, *Klebsiella pneumoniae*, and *Escherichia coli*, and tube images processed by CRISPR/Cas14 detection system; Right: Fluorescence bar charts of CRISPR/Cas14 detection system recognizing *H. pylori*, *Vibrio parahaemolyticus*, *Salmonella*, *Klebsiella pneumoniae*, and *Escherichia coli*. (E) Left: Gel images of ultrafast PCR amplification of wild-type *H. pylori* strains and *H. pylori* levofloxacin-resistant strains, and tube images processed by CRISPR/Cas14 detection system; Right: Fluorescence bar charts of CRISPR/Cas14 detection system recognizing wild-type *H. pylori* strains and *H. pylori* levofloxacin-resistant strains. Data are presented as the mean ± SD (n = 3), $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

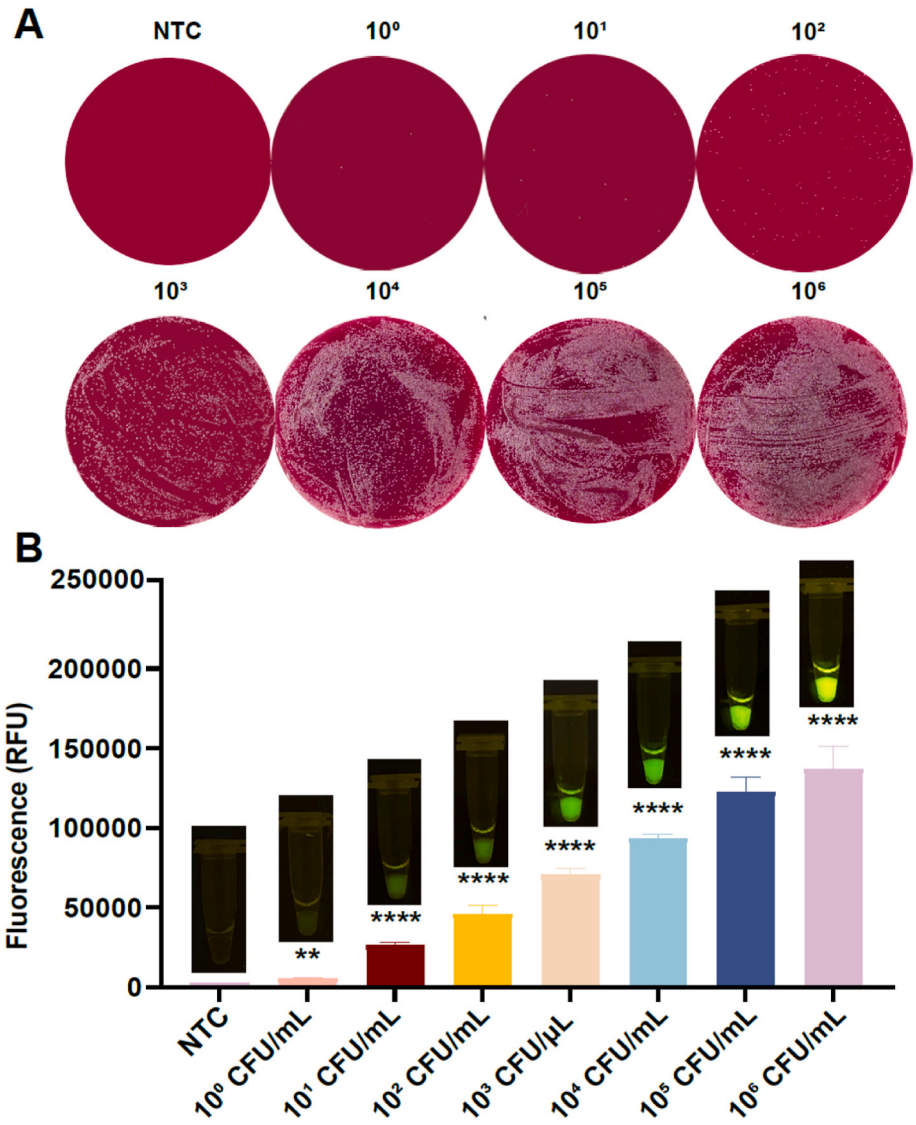
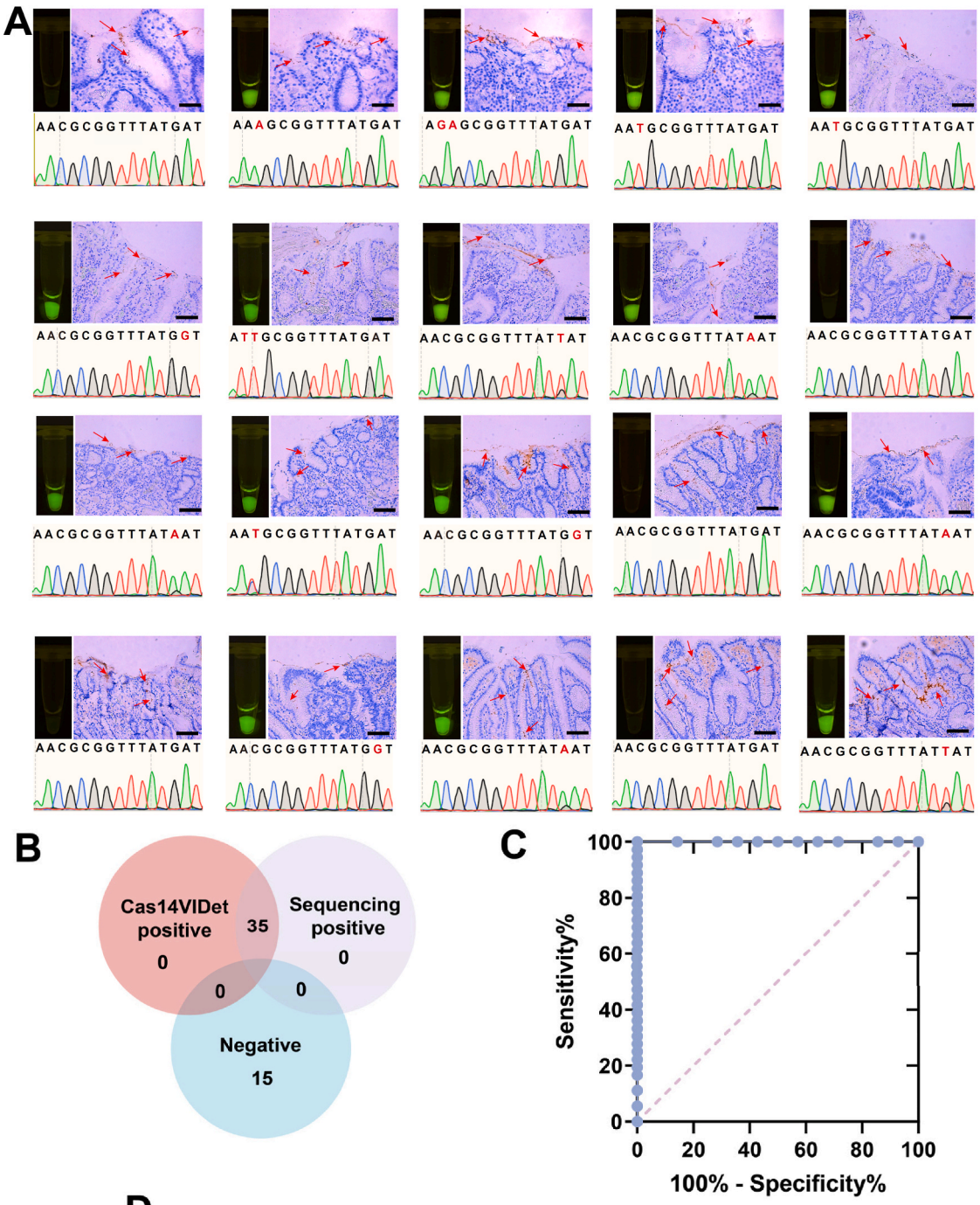


Fig. 5. Sensitivity Testing of the Cas14VIDet Detection System. (A) The *H. pylori* colony counts were demonstrated using the standard plate count method at concentrations of 10^0, 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 CFU/mL, respectively. (B) Fluorescence tube images and fluorescence bar graph showing the Cas14VIDet system identifying different concentrations of *H. pylori*. Data are presented as the mean ± SD (n = 3), $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

further would complicate the design of the Cas14VIDet system and raise costs (Mohr et al., 2016). Currently, there are highly efficient isothermal amplification techniques such as loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), and rolling circle amplification (RCA), which can rapidly amplify target genomes in a short period of time and can be easily integrated with CRISPR systems to achieve one-pot or even one-step reactions. However, compared to the ultrafast PCR amplification system developed in our study, LAMP still has drawbacks such as complex primer design, intricate reaction components, and lower specificity (Gines et al., 2020; Sharma et al., 2021; Kim et al., 2023). Additionally, while LAMP's reaction time is significantly shorter than that of conventional PCR, its isothermal reaction typically requires around 30 min (Rolando et al., 2024). Other isothermal amplification methods, such as RPA and RCA, also require longer amplification times and involve more complex reaction systems (Niemz et al., 2011). In contrast, our ultra-rapid PCR system reaches a detectable state within 5 min, and compared to the previously mentioned methods, the system we developed is significantly simpler and more convenient. Therefore, we opted to use the ultrafast PCR system in this study.

Currently, CRISPR detection tools include Cas9, Cas12, Cas13, and Cas14(Wang et al., 2023). Unlike other researchers who typically choose CRISPR/Cas13a, CRISPR/Cas12a, or CRISPR/Cas9 technologies, we opted CRISPR/Cas14 for several reasons. Cas13 targets RNA, and its detection process may require complex preprocessing, which increases operational complexity and time (Liu et al., 2017). Cas9 lacks trans-cleavage activity, which limits its ability to amplify and output signals, reducing its advantages in detection (Zetsche et al., 2015; Qiu et al., 2021). Cas12 is commonly used, but its dependence on the presence of a PAM sequence limits its application range (Wang et al., 2023). In contrast, Cas14 does not require a PAM, enabling precise gene targeting, which offers greater advantages for clinical translation.

In clinical practice, antibiotic susceptibility testing for *H. pylori* commonly relies on methods such as disk diffusion, E-test, and agar dilution, with some well-equipped hospitals employing RT-PCR. Compared to these methods, Cas14VIDet offers substantial advantages and improvements. First, our method uses CRISPR, which allows for the detection of SNPs through gRNA specifically targeting the sequence, greatly enhancing detection specificity (Kaminski et al., 2021). Our tests on clinical samples revealed that this method is highly accurate and sensitive, even surpassing first-generation sequencing techniques. Second, the total detection time for Cas14VIDet is no more than 10 min, and



D

Methods		Sequencing		Total
		Positive	Negative	
Cas14VIDet	Positive	35	0	35
	Negative	0	15	15
	Total	35	15	50
Sensitivity: 100%; Specificity: 100%; Accuracy: 100%; PPV: 100%; NPV: 100%;				

Fig. 6. Robust performance of the Cas14VIDet assay for detection of *H. pylori* levofloxacin-resistant gene in clinical specimens. (A) Representative images of clinical *H. pylori* sample detection. The left image shows the fluorescence results of detecting the levofloxacin resistance gene using Cas14VIDet, captured by a smartphone. The right image is an immunohistochemistry staining of *H. pylori* in gastric tissue, with red arrows indicating the presence of *H. pylori* infection. Below is the bacterial sequencing result, with red marks indicating the mutation sites of the resistance gene. Bars represent 100 μm. (B) Venn diagram showing detection accuracy analysis of Cas14VIDet and sequencing. (C) ROC curve of Cas14VIDet for diagnosing levofloxacin resistance genes of *H. pylori*. (D) The negative predictive value (NPV), positive predictive value (PPV), sensitivity, and specificity of Cas14VIDet in diagnosing levofloxacin resistance genes of *H. pylori*.

its operation is simple, requiring no complex instruments or highly trained personnel (Table S1). This significantly shortens the traditional time required for antibiotic resistance testing in *H. pylori*, allowing timely and targeted treatment after diagnosis, reducing the need for follow-up visits, and minimizing waste of medical resources. Third, Cas14VIDet integrates ultra-fast PCR and CRISPR-Cas14 into a single workflow, including initial amplification and targeted cleavage, with an oil barrier isolating the two systems at the start of the reaction. This minimizes the risk of aerosol contamination during the transition from one stage of amplification to the next, thereby reducing the risk of nucleic acid contamination due to manual handling or evaporation, and further improving the accuracy of the Cas14VIDet assay.

However, this study has certain limitations. First, we only tested H. pylori resistance mutation sites for levofloxacin, and it remains unclear whether this method is applicable to detecting resistance to other commonly used antibiotics, such as clarithromycin. Nonetheless, theoretically, the Cas14VIDet system we constructed is capable of detecting all types of base mutations, and future research will include tests designed to detect clarithromycin resistance genes. Second, we only tested 50 samples, which is a relatively small sample size. In future studies, we plan to expand the sample size to further validate the sensitivity and specificity of this detection technology.

5. Conclusion

This study developed an ultrasensitive, interference-resistant, one-pot visual nucleic acid analysis method, designed for bacterial resistance gene detection and clinical diagnostics. Cas14VIDet can cleave target single-stranded DNA without the need for restrictive sequences and exhibits specificity for SNP identification with high-fidelity SNP detection capabilities. The entire detection process can be completed within 10 min, showing strong ability to distinguish between non-resistant bacteria and different types of resistant bacteria. Compared to current clinical methods for resistance detection, Cas14VIDet significantly reduces testing time and cost, while maintaining sensitivity comparable to qRT-PCR. This method can be integrated with simple, portable, and cost-effective reporting devices such as fluorescence readers or blue light lamps, allowing for instant result output. In summary, Cas14VIDet is a versatile resistance gene detection technology characterized by high specificity, sensitivity, cost-effectiveness, portability, and ease of use. It has the potential for widespread application in low-resource settings, offering promising support for personalized *H. pylori* eradication therapy worldwide. In the future, expanding sample testing to further evaluate its detection performance, along with assessing the method's ability to detect *H. pylori* clarithromycin resistance genes, will be crucial for translating this technology from basic research to clinical application. Additionally, integrating nucleic acid extraction, amplification, and CRISPR detection into a single chip to achieve a streamlined, rapid sample-to-result detection process will make it more suitable for on-site and real-time diagnostics.

CRediT authorship contribution statement

Yongkang Lai: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. **Kaiming Guo:** Data curation, Formal analysis, Methodology. **Chunping Zhu:** Data curation, Investigation, Methodology. **Yanwei Lv:** Methodology, Validation. **Hang Wu:** Methodology, Validation. **Liang Zhang:** Methodology, Validation. **Mengni Jiang:** Methodology, Validation. **Yanan Pang:** Methodology, Validation. **Jiulong Zhao:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision. **Rui Wang:** Conceptualization, Funding acquisition, Project administration, Writing – review & editing. **Zhaoshen Li:** Conceptualization, Project administration, Supervision, Writing – review & editing.

Ethics statement

All clinical samples were provided by the Department of Gastroenterology, the First Affiliated Hospital of Naval Medical University, and the study was approved by the Ethics Committee of the First Affiliated Hospital of Naval Medical University (CHEC2021-131), with verbal notification of obtaining consent from all patients involved.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2024.116884.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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