



Article

Evaluation of In Vitro Efficiency of Ciclopirox Against *Yersinia pestis* and *Francisella tularensis*

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Abstract

Yersinia pestis and *Francisella tularensis* are Tier-1 pathogens with high interest for biodefense and public health. Evaluating the antibacterial activity of repurposed drugs against these high-priority pathogens is a key element in the ongoing effort to develop diversified antimicrobial strategies. Drug repurposing offers a cost-effective and time-efficient approach to address antibiotic resistance by identifying new applications for existing therapeutics. In this study, we demonstrate in vitro antibacterial effect of the antifungal agent ciclopirox and offer this drug as a potential antibacterial treatment. Ciclopirox in vitro activity was previously reported against various Gram-negative bacteria, including resistant strains, primarily through iron chelation that disrupts key metabolic pathways and virulence mechanisms. Additionally, it exhibits antibiofilm activity and can potentiate the efficacy of certain antibiotics. Our findings reveal that ciclopirox effectively inhibits the in vitro growth of fully virulent strains of *Y. pestis* and *F. tularensis*, as well as avirulent isolates, including avirulent mutants that their wild-type susceptibility was reduced through selection to MIC levels defining them as “nonsusceptible” to ciprofloxacin (*Y. pestis* Kim53Δ70Δ10 and *F. tularensis* LVS) and doxycycline (LVS), or resistant to doxycycline (Kim53Δ70Δ10) according to CLSI interpretive criteria. Additionally, prolonged exposure of *Y. pestis* and *F. tularensis* to sub-MIC and MIC concentrations of ciclopirox did not lead to an increase in observed MIC during the study period. These results highlight ciclopirox as a potential candidate for treatment alternative, combined with other antibiotic substances or repurposed drugs against these bacterial threats.

Keywords: ciclopirox; drug repurposing; antibiotics-resistance; *Yersinia pestis* and *Francisella tularensis*

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1. Introduction

Antibiotic resistance causes a serious and growing threat to global healthcare systems. This challenge is rapidly escalating into a crisis, largely fueled by the overuse and misuse of antibacterial drugs (such as treating viral infections, widespread use in agriculture, animal husbandry, etc.). At the same time, there is limited incentive for pharmaceutical companies to invest in developing new antibiotics, as these are often considered less profitable compared to other treatments (such as long-term or chronic disease drugs). As a result, over the past decade, only a small number of new antibiotic compounds have been approved by regulatory agencies such as the Food and Drug Administration (FDA) and European Medicines Agency (EMAs) [1,2].

The CDCs (Centers for Disease Control and Prevention) have categorized several bacteria as posing alarming, significant, and threats to healthcare systems. Many of these already lead to significant burdens (economic and clinical) on healthcare systems around the world as well as on patients and their families. Furthermore, antibiotics resistance in these bacteria can be readily acquired synthetically in the laboratory [3].

Drug repurposing has emerged in recent years as a highly studied therapeutic approach, serving as an effective strategy for re-evaluating clinically approved and safe drugs for the treatment of new medical conditions. This approach is characterized by lower development costs, shortened development times, and improved safety profiles compared to de novo drug development [4]. Specifically, there is extensive scientific literature on drug repurposing aimed at combating bacteria in general, and antibiotic-resistant bacteria in particular.

Ciclopirox is an antifungal drug approved for topical use in humans for many years. As evidenced by the literature, this drug is a suitable candidate for evaluation as a repurposed drug to combat antibiotic-resistant bacteria. Ciclopirox exhibits direct antibacterial activity against clinical isolates of the Gram-negative bacteria *A. baumannii*, *E. coli*, *K. pneumoniae*, and *P. aeruginosa*, with sensitivity in the range of MIC = 5–15 µg/mL (regardless of whether the bacterium is susceptible or resistant to other antibiotics). Its mechanism of action (specifically in *E. coli*) involves inhibiting LPS synthesis and galactose metabolism (two pathways critical for virulence) [5,6]. A broader direct antibacterial mechanism of ciclopirox, which appears to be broad-spectrum (antifungal, antibacterial, and antiviral), involves iron chelation [7,8]. Furthermore, iron chelation may have indirect implications, as iron is a critical factor for bacterial virulence. The drug may also be effective against biofilms [9] and can reduce the expression and activity of virulence factors [10]. Synergism of ciclopirox with polymyxin B has been observed against Gram-negative bacteria, including resistant strains (*E. coli*, *A. baumannii*, and *P. aeruginosa*) [11].

Although ciclopirox is approved only for topical treatment, clinical trials have been conducted over the past decade evaluating its efficacy (repurposing for cancer treatment) via oral administration (www.clinicaltrials.gov—NCT00990587, NCT05647343). Additionally, intravenous administration of the prodrug of ciclopirox (fosciclopirox) appeared to be well tolerated in clinical trials (www.clinicaltrials.gov—NCT03348514, NCT04956042) [12]. The favorable safety data, coupled with the lack of evidence for the development of resistance in fungi [13–15] and to date, in bacteria [7], may serve as criteria for developing treatments for resistant bacteria.

In the study presented here, we demonstrate that ciclopirox exhibits in vitro antibacterial activity against Tier-1 Gram-negative pathogens *Y. pestis* (virulent strain Kim53) and *F. tularensis* (virulent strain Schu S4), as well as against the respective attenuated *Y. pestis* derivatives Kim53Δ70Δ10, Kim53Δ70Δpgm, and Kim53Δ10Δpgm, and the *F. tularensis* live Vaccine Strain (LVS). Susceptibility to ciclopirox was not affected by mutations that reduced the wild-type sensitivity of Kim53Δ70Δ10 or LVS to first choice treatment drugs, namely doxycycline or ciprofloxacin to levels categorizing them as “nonsusceptible” or resistant according to CLSI interpretive guidelines [16]. These findings warrant further investigation into the therapeutic potential of this compound against high-consequence pathogen.

2. Results

2.1. Inhibition of Bacterial Growth

Determination of the ciclopirox MICs against strains of *F. tularensis* and *Y. pestis* was conducted by microdilution assays according to CLSI guidelines [16] (Figure 1). The MIC obtained at 48 h of incubation for *F. tularensis* Schu S4 was in the range of 3–6 µg/mL (44% MIC = 3 µg/mL, 56% MIC = 6 µg/mL [$n = 16$]), and for LVS in the range of 1.5–6 µg/mL

(50% MIC = 1.5, 43% MIC = 3 $\mu\text{g/mL}$, 7% MIC = 6 $\mu\text{g/mL}$ [$n = 30$]). These results indicate that the LVS strain is highly similar to the virulent Schu S4 strain in respect to ciprofloxacin sensitivity, as their MIC ranges differ by about one 2-fold dilution (Figure 1a,b respectively). Similar MIC results were obtained for the virulent and avirulent derivatives of *Y. pestis*. The MIC obtained after 24 h for the virulent strain Kim53 was 12 $\mu\text{g/mL}$ [$n = 6$], while for the avirulent derivative Kim53 $\Delta 70\Delta 10$ the MIC was in the range of 6–12 $\mu\text{g/mL}$ (60% 6 $\mu\text{g/mL}$, 40% 12 $\mu\text{g/mL}$ [$n = 20$]) (Figure 1c,d respectively). MICs of 12 $\mu\text{g/mL}$ were obtained also for the Kim53 $\Delta 70\Delta pgm$ and Kim53 $\Delta 10\Delta pgm$ derivatives, indicating that the absence of the *pgm* locus or the virulence plasmids pCD1 and pPCP1 (all required virulence factors in vivo), do not lead to a measurable effect on MICs by iron deprivation of ciprofloxacin in vitro.

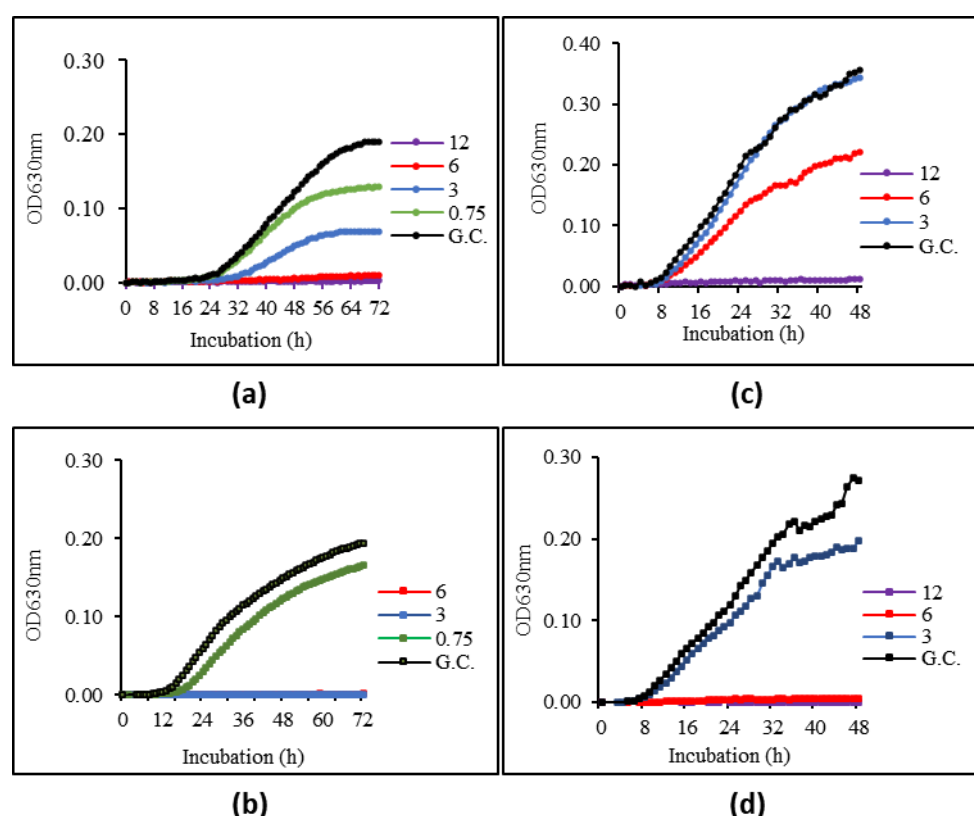


Figure 1. Determination of ciprofloxacin MICs for *F. tularensis* and *Y. pestis* strains was conducted in 96-well microtiter plates. *F. tularensis* strains were incubated with the indicated ciprofloxacin concentrations in standard liquid medium (HLMHI) for 72 h at 37 °C, to assess growth and determination of the MIC at 48 h (a) LVS (b) Schu-S4. *Y. pestis* strains were incubated with the indicated ciprofloxacin concentrations in standard CAMHB medium for 48 h at 28 °C to assess growth and determination of the MIC at 24 h (c) Kim53 (d) Kim53 $\Delta 70\Delta 10$. Growth was monitored hourly (OD_{630nm}) using plate readers (c) Kim53 (d) Kim53 $\Delta 70\Delta 10$. All data represent the mean of 3–6 replicates and tests were repeated in three independent days. Curve colors indicate ciprofloxacin concentrations ($\mu\text{g/mL}$). G.C. is the growth control in the test medium without ciprofloxacin.

2.2. Minimal Bactericidal Concentration

Minimal Bactericidal Concentration (MBC) of ciprofloxacin to *F. tularensis* (LVS) was determined by plating a standard concentration of bacterial culture (ca. 5×10^4 cfu/well) into microplate wells containing a series of twofold dilutions of ciprofloxacin in HLMHI and incubating at 37 °C under conditions identical to the microdilution MIC assay. The CFU counts of the *F. tularensis* culture was measured at time zero (start of the experiment), and after 48 h of incubation. Bacterial survival in the wells was assessed by preparing serial

dilutions and plating for viable plate counts on mCHA plates. The MBC value was defined as the minimal concentration at which a 3-log reduction (99.9% kill or $\leq 0.1\%$ survival) in the number of surviving bacteria occurs relative to time zero. The percentage of LVS surviving bacteria is presented in Figure 2a. The ciprofloxacin concentration at which a 3-log reduction was observed at the time of MIC determination (48 h, MIC = 3 $\mu\text{g/mL}$) relative to the initial bacterial count (at time zero) was MBC = 24 $\mu\text{g/mL}$. At this concentration, 0.01% survived, and this is the first concentration where less than 0.1% survived (>3 log reduction) [17]. The MBC/MIC ratio for this bacterium at 48 h is eight. This value is >3 MIC multiplications, thus indicating bacteriostatic activity of ciprofloxacin against *F. tularensis*, although only by a factor of two above the bactericidal “cutoff” definition. However, it should be noted that for slow-growing organisms, further incubation should be allowed (as doubling time and killing may take longer), and indeed, when checked after 56, 64 and 72 h, the MBC/MIC ratio drops to the bactericidal definition range of MBC/MIC = 4 (see time-kill curves in Figure 3a).

In a similar assay the MBC of ciprofloxacin was examined against *Y. pestis* Kim53 Δ 70 Δ 10 in CAMHB at 28 °C. The MIC at 24 h was 12 $\mu\text{g/mL}$. Viable plate counts revealed that only at a concentration of 384 $\mu\text{g/mL}$ there was a reduction in more than 3 logs, to less than 0.1% relative to the initial inoculum at time zero (in this case, a reduction to 0.02%) (Figure 2b). Therefore, this drug MBC/MIC = 32 was clearly found to show bacteriostatic activity for Kim53 Δ 70 Δ 10 bacteria.

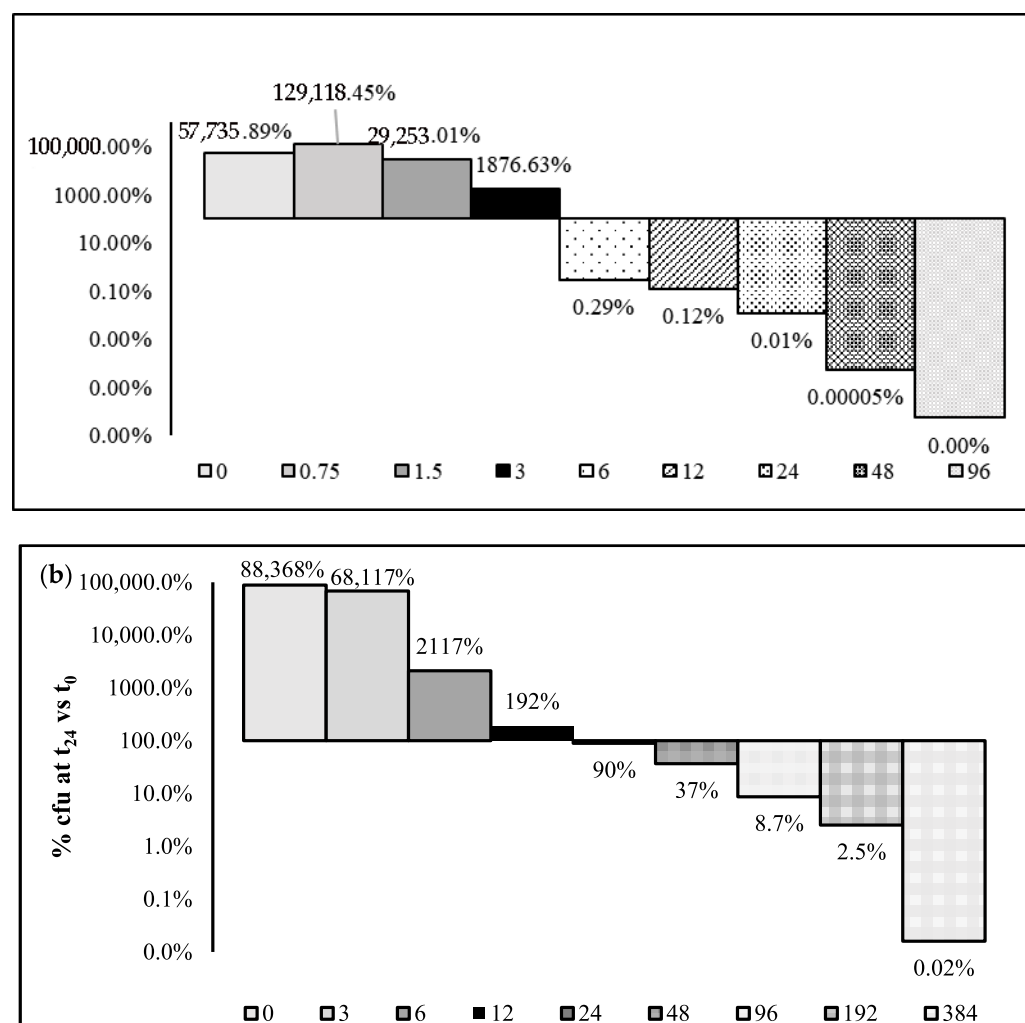


Figure 2. (a) Percentage of LVS survival after 48 h exposure to the indicated ciprofloxacin concentration. A standard LVS inoculum was incubated in triplicates in microplates containing HLMHI medium

with ciprofloxacin (patterns indicate concentrations in $\mu\text{g/mL}$) at 37°C . After 48 h, MICs were determined ($\text{OD}_{630\text{nm}}$ and visual check), and viable counts on mCHA plates quantified survivals. Percent survival is shown relative to the initial inoculum counts. **(b)** Percentage of *Y. pestis*- Kim53 Δ 70 Δ 10 survival after 24 h exposure to ciprofloxacin. A standard *Y. pestis* inoculum was incubated at 28°C in CAMHB with ciprofloxacin (patterns indicate concentrations in $\mu\text{g/mL}$). After 24 h, MIC was determined ($\text{OD}_{630\text{nm}}$ and visual check), and viable counts on BHIA plates measured survivals. Percent survival is shown relative to the initial inoculum counts.

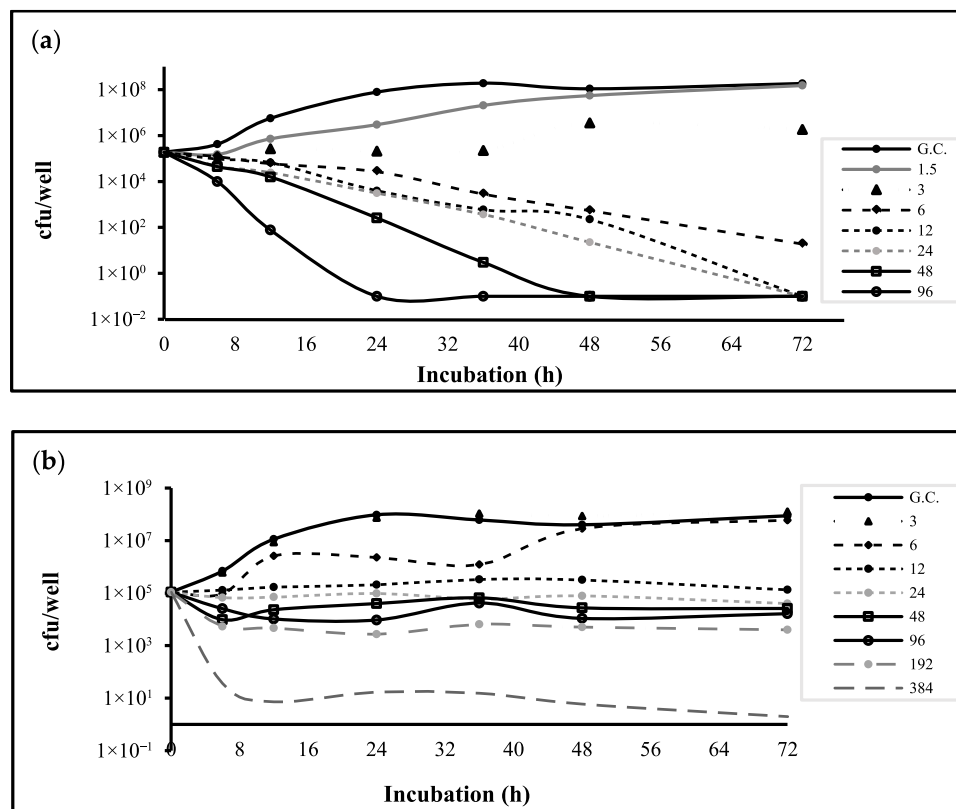


Figure 3. Characterization of ciprofloxacin TKC curves against LVS and Kim53 Δ 70 Δ 10. **(a)** A standard inoculum of LVS was incubated in microplates at 37°C in HLMHI containing ciprofloxacin. **(b)** A standard inoculum of *Y. pestis* was incubated in microplates at 28°C in CAMHB containing ciprofloxacin. At each time point, two duplicates of all concentrations were sampled, pooled and serially diluted in PBS for viable CFU assessment by plate counts. Line styles in the diagram indicate ciprofloxacin concentrations in $\mu\text{g/mL}$. Similar results were obtained in tests from three independent days.

2.3. Time Kill Curves

The time-kill (TKC) measurements were conducted in multiple replicates of the microdilution MIC assay, by sampling two replicates at each indicated time point for CFU counts in serial 10-fold dilutions. The TKC for LVS (Figure 3a) showed that after 48 h (MIC = $3 \mu\text{g/mL}$), a ciprofloxacin concentration of $24 \mu\text{g/mL}$ was the minimal concentration to produce a reduction in more than 3 log units, again defining this concentration as the MBC as shown above (see Figure 2a). Furthermore, as incubation progressed towards 72 h, a reduction of over three orders of magnitude was observed also at lower concentrations of $12 \mu\text{g/mL}$ and even $6 \mu\text{g/mL}$ (thus lowering the $\text{MBC}_{72\text{h}}/\text{MIC}$ ratio to 2). This phenomenon supports the conclusion that ciprofloxacin's mode of action against the *F. tularensis* bacterium can be considered as bactericidal.

For *Y. pestis* Kim53 Δ 70 Δ 10 (Figure 3b), the ciprofloxacin MIC was $12 \mu\text{g/mL}$, which remained consistent throughout the incubation. Ciprofloxacin concentrations below the MIC

(0, 3, 6 µg/mL) allowed for bacterial proliferation. However, even at concentrations significantly above the MIC (24–192 µg/mL), the reduction in bacterial count was limited to less than two orders of magnitude. A substantial reduction of approximately four orders of magnitude, defining the MBC, was only observed at 384 µg/mL. With an MBC/MIC ratio not lower than 32, ciclopirox acts as a bacteriostatic growth inhibitor against Kim53Δ70Δ10.

2.4. Susceptibility to Ciclopirox in Ciprofloxacin and Doxycycline Reduced Susceptibility Isolates

Antibacterial activity of ciclopirox was investigated in avirulent isolates of LVS and Kim53Δ70Δ10 that were selected for reduced susceptibility to either ciprofloxacin or doxycycline. The term reduced susceptibility is used to describe the range of non-WT MIC range for *F. tularensis* that can only be defined as susceptible (S) to ciprofloxacin and doxycycline when MICs are ≤0.5 µg/mL and ≤4 µg/mL, respectively, while higher MICs suggestive of a “nonsusceptible” category cannot be used to define intermediate (I) or resistant (R) categories (CLSI, M45). Similarly, *Y. pestis* is defined as susceptible to ciprofloxacin for MICs ≤0.25 µg/mL, while higher MIC values cannot be used to interpret the “nonsusceptible” strain as intermediate or resistant. However, *Y. pestis* has defined categories for tetracyclines, with MIC interpretive criteria of ≤4, 8 and >16 µg/mL for S, I, and R categories respectively.

As shown in Table 1, ciclopirox’s MICs remained within the 6–12 µg/mL range across a broad spectrum of *Y. pestis* isolates exhibiting reduced susceptibility to ciprofloxacin (MIC range 0.008–16 µg/mL) or resistance to doxycycline (up to 32 µg/mL) as compared to the parental strain (Kim53Δ70Δ10). These findings align with previous research by Carlson-Banning et al. [7], who demonstrated that ciclopirox’s MIC remained within a narrow range of 5–15 µg/mL against thirty clinical *E. coli* isolates, half of which were highly resistant to ciprofloxacin (MIC = 50–500 µg/mL). Similar consistency was observed in *A. baumannii*, *K. pneumoniae*, and *P. aeruginosa* (8–9 resistant clinical isolates of each) [7].

Similarly, the MICs of ciclopirox against *F. tularensis* LVS isolates with reduced susceptibility to ciprofloxacin remained within a range of 1.5–3 µg/mL but demonstrated a up to one doubling dilution difference in the MIC range of the isolates with reduced susceptibility to doxycycline (Table 2). As these deviations did not occur in all replicates, they do not seem to represent a mechanistic relevance to ciclopirox susceptibility.

Table 1. Determination of ciclopirox, ciprofloxacin, and doxycycline MICs in reduced-susceptibility *Y. pestis* Kim53Δ70Δ10 isolates.

Reduced Susceptibility Antibiotics	MIC (µg/mL)		Isolate Number
	Ciclopirox	Antibiotics	
Ciprofloxacin	6–12	0.008–0.016	* YP-1
	6–12	0.25	YP-2
	12	0.5	YP-3
	12	8	YP-4
	6	8	YP-5
	12	8	YP-6
	12	16	YP-7
Doxycycline	6–12	0.25–0.5	YP-1
	12	8–16	YP-8
	6	32	YP-9

* Isolate YP-1 has the wild-type susceptibility of the parental Kim53 strain. Ciclopirox, ciprofloxacin and doxycycline MICs were determined by microdilution for 24 h in CAMHB at 28 °C. Results represent the MIC ranges obtained in three independent tests.

Table 2. Determination of ciclopirox, ciprofloxacin, and doxycycline MICs in reduced-susceptibility LVS isolates.

Reduced Susceptibility Antibiotics	MIC (µg/mL)		Isolate Number
	Ciclopirox	Antibiotics	
Ciprofloxacin	1.5–3	0.008–0.016	* LVS
	1.5–3	0.4–0.8	LVS-2
	1.5–3	0.8–1.6	LVS-3
Doxycycline	1.5–6	0.125–0.25	LVS
	0.75–1.5	1–2	LVS-4

* LVS is the wild-type parental strain used for generating the reduced-susceptibility isolates (for *F. tularensis* there are no CLSI categories other than susceptible or “nonsusceptible” for doxycycline and ciprofloxacin). Ciclopirox, ciprofloxacin, and doxycycline MICs were determined by microdilution for 48 h in HLMHI at 37 °C. Results represent the MIC ranges obtained in three independent tests.

2.5. The Proposed Mechanism of Antibacterial Effect of Ciclopirox Is Iron Chelation

Other studies indicate that ciclopirox’s primary antibacterial (and antifungal) mechanism of action involves iron chelation [7,8]. Unlike other chelators that bind extracellular iron (e.g., deferoxamine) or reduce ferric to ferrous iron (e.g. triapine), ciclopirox is thought to bind intracellular iron [18], thereby inhibiting iron-dependent enzymes by blocking iron incorporation [19].

To elucidate whether the antibacterial mechanism of action of ciclopirox works similarly in the Tier-1 bacterial agents, we investigated whether increasing iron concentrations in the medium would inhibit or abolish ciclopirox’s growth inhibitory activity against *Y. pestis* (Kim53, Kim53Δ70Δ10, Kim53Δ70Δpgm and Kim53Δ10Δpgm) and *F. tularensis* (Schu S4 and LVS). For this purpose, these bacteria were incubated under standard microdilution conditions in the appropriate medium for ciclopirox MIC determination, while replica of the same MIC assays was incubated with various concentrations of FeSO₄ to determine iron’s effect on the MICs. As shown in Table 3, adding increasing concentrations of FeSO₄ led to a dramatic, dose-dependent increase in ciclopirox’s MIC values (16–≥64 fold compared to ciclopirox alone without FeSO₄).

Table 3. Determination of ciclopirox MIC in the presence of the indicated iron concentrations against *F. tularensis* and *Y. pestis* virulent and avirulent isolates, with or without the 102 kb *pgm* locus, pPCP1 or pCD1 virulence plasmids.

FeSO ₄ (µM)	Ciclopirox MIC (µg/mL)					
	<i>Y. pestis</i> (Kim53)	<i>Y. pestis</i> (Kim53Δ70Δ10)	<i>Y. pestis</i> (Kim53Δ10Δpgm)	<i>Y. pestis</i> (Kim53Δ70Δpgm)	<i>F. tularensis</i> (Schu S4)	<i>F. tularensis</i> (LVS)
0	12	12	12	12	3–6	3
3.125	24	12–24	24	24	12	6–12
6.25	24–48	24	24	24	12–24	12
12.5	48	48	48	48	48	24–48
25	96	48–96	48	48	48–96	48–96
50	192	96–192	48–96	48–96	96–192	96–192
100	192–>192	192	192	192	192–>192	192–>192
	<i>n</i> = 3	<i>n</i> = 6	<i>n</i> = 3	<i>n</i> = 3	<i>n</i> = 5	<i>n</i> = 5

The above results indicate that the addition of iron (as FeSO₄) titrates the antibacterial iron chelating activity of ciclopirox, while the addition of iron itself had little to no effect on bacterial growth rate in the growth control wells (Figure 4), indicating that the observed phenomenon is due to the drug’s effect being suppressed by addition of iron.

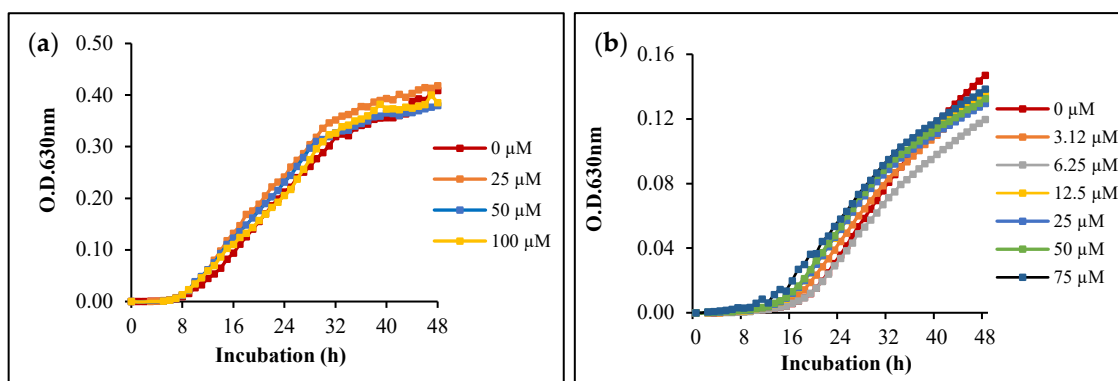


Figure 4. Effect of iron on bacterial growth in presence of FeSO₄. (a) *Y. pestis* (Kim53Δ70Δ10) was incubated in microdilution assay conditions in the presence of the indicated concentrations of FeSO₄ added to CAMHB at 28 °C. (b) *F. tularensis* (LVS) was incubated in microdilution conditions in HLMHI containing the indicated concentrations of FeSO₄ at 37 °C. The growth curves are the growth controls without ciclopirox from a representative assay summarized in Table 3. Similar results were obtained with the avirulent *Y. pestis* Kim53-derivatives Kim53Δ10Δpgm, Kim53Δ70Δpgm and the Schu S4 virulent strain.

Cho and Kim [20] demonstrated that while selective pressure rapidly induces resistance of *E. coli* ATCC 25922 to azidothymidine (<2 days), the same pressure does not result in a significant increase in MIC was observed during the study period (growth for 25 days). These findings are consistent with the lack of evidence in the literature for the development of resistance to ciclopirox. Accordingly, we investigated whether this observation also applies to *Y. pestis* and *F. tularensis*. For this purpose, we exposed these strains to selective pressure of either ciclopirox or ciprofloxacin (Figure 5). Our results show that while exposure to gradually increasing concentrations of ciprofloxacin in liquid medium led to the emergence of resistance (manifested by a rapid and marked increase in ciprofloxacin MIC values for both strains), the selective pressure did not result in any increase in ciclopirox MIC during our study (cumulative exposures of 24 and 36 days for *Y. pestis* and LVS respectively). The lack of rise in MIC within the limited exposure cycles probably represents multiple intracellular iron-dependent targets and processes that cannot be mutually mutated to gain resistance to iron deprivation (Figure 5).

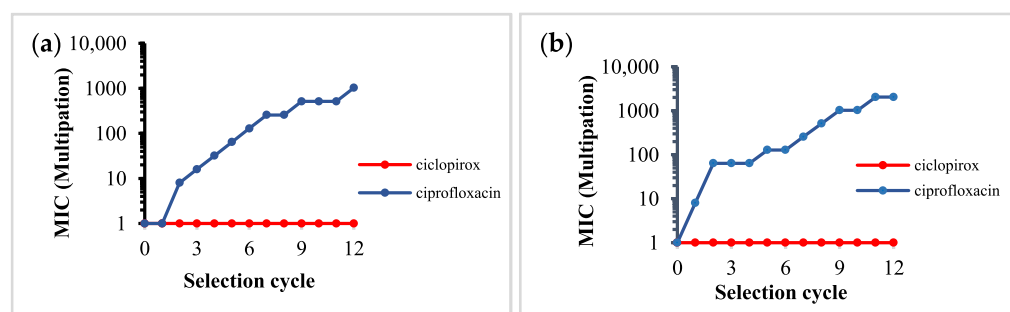


Figure 5. Selective pressure—changes in MIC values during selection cycles of (a) *Y. pestis* Kim53 Δ70Δ10 and (b) *F. tularensis* LVS. Bacteria were exposed in cycles to increasing concentrations of ciprofloxacin (0.5×, 1×, 2×, 4× MIC) or ciclopirox (0.25×, 0.5×, 1×, 2× MIC) in 5 mL of appropriate medium. Cycles continued for two days for *Y. pestis* and for 3 days for *F. tularensis*. For ciprofloxacin, the next cycle was inoculated from the highest concentration that showed growth (0.5× new MIC) for both strains. For ciclopirox, *Y. pestis* showed growth only in 0.5× MIC which was used for the next cycle. For LVS, although some low turbidity could be observed in the “0.5× MIC”, live bacteria

could not be recovered from this concentration. Only “0.25× MIC” concentration produced live bacteria that were used in the next exposure cycle (which repeatedly resulted in growth in the 0.25× MIC, low turbidity and no live bacteria in the 0.5× MIC, and no turbidity and no live bacteria in the 1× or 2× MIC concentrations).

3. Discussion

In this study, we demonstrated that ciclopirox exhibits an in vitro antibacterial effect against both virulent and non-virulent strains of *Y. pestis* and *F. tularensis*. Our data suggest that despite the physiological differences between strains, accounting to the growth rates according to standardized CLSI-based guidelines (MIC determination at 24 h for *Y. pestis* and 48 h for *F. tularensis*), the drug’s mechanism of action (primarily iron chelation of Fe³⁺ and other polyvalent metal ions) targets fundamental metabolic processes that are essential for both pathogens. The antibacterial effect is more potent against *F. tularensis* LVS (MIC = 1.5–3 µg/mL) and Schu S4 (MIC = 3–6 µg/mL), compared to *Y. pestis* Kim53 (MIC = 12 µg/mL). Additionally, MBC and TKC results showed that ciclopirox is on the border definition between bactericidal and bacteriostatic activity against *F. tularensis* (MBC/MIC ratio = 4–8) but definitely bacteriostatic against *Y. pestis* (MBC/MIC = 32). These results indicate that repurposing of ciclopirox as treatment alternative would probably have to rely on a combination with other antibiotics or repurposed drugs.

Over forty years ago, Sakurai and colleagues [18] showed in fungi that the drug binds irreversibly to intracellular components (intracellular concentrations are 200-fold higher than extracellular levels, with over 97% of the drug bound to cellular organelles and components, while only a minor fraction remains free in the cytoplasm, all without intracellular metabolism or degradation). Therefore, it is unlikely that the drug serves as a substrate for efflux pumps and would thus not be rapidly expelled from the cell. Moreover, the major mechanism of action of ciclopirox is iron and possibly other polyvalent metal ions chelation, which disrupts multiple iron- and metal-dependent processes such as metalloenzymes involved in energy metabolism (e.g. catalase, peroxidases, and cytochromes), DNA repair and replication, oxidative stress pathways (disruption of oxidative stress defense, accumulation of ROS, impaired ATP production and failure of essential biosynthetic pathways). Unlike classical antibiotics for which development of resistance is usually evident within few years of clinical use, the co-disruption of multiple processes suggests a very low likelihood of resistance development or cross-resistance with classical bacterial resistance mechanisms. These factors support our findings that the MIC of ciclopirox does not change also in the avirulent *Y. pestis* and *F. tularensis* strains selected for reduced susceptibility or resistance to conventional antibiotics (ciprofloxacin and doxycycline). Furthermore, although exposure was limited to 12 cycles, we were unable to induce any change in ciclopirox MICs through the selective pressure in both of these bacteria (unlike ciprofloxacin). This observation aligns with similar studies showing that selective pressure did not result in ciclopirox resistance in various Gram-negative bacteria, in contrast to the resistance that develops under selection with antibiotics or other drugs such as ciprofloxacin [7] or azidothymidine [20]. However, further research should include comprehensive genomic and transcriptomic studies over extended passage periods, which are essential to fully determine the long-term resistance profile potential of ciclopirox.

Our results support iron chelation as a principal antibacterial mechanism of ciclopirox, consistent with the evidence summarized in the review by Shen and Huang [21]. Specifically, supplementation of the growth medium with iron (FeSO₄) fully reversed the antibacterial effect of ciclopirox against both *F. tularensis* and *Y. pestis*. This result implies a broad mechanism of action that may extend to other antibiotic-resistant bacterial species. Because iron availability is a pivotal factor during infection, strategies that modulate the

host–pathogen competition for this metal have the potential to favor the host. To this end, siderophores may be chemically modified to serve as iron-chelating agents, or existing iron chelators can be further optimized to potentiate immune-mediated restriction of bacterial growth. Additionally, siderophores have recently gained attention as targeted delivery platforms for directing antibiotics into pathogenic bacteria [22]. In the context of virulent *Y. pestis*, it is noteworthy that the drug's effect on iron availability may independently help control the pathogen, as upregulation of iron storage has been associated with reduced virulence [23].

Importantly, beyond its direct antibacterial effects, ciclopirox has immunomodulatory properties [15], including inhibition of NLRP3 inflammasome activation [24] and antioxidant activity [25]. Furthermore, ciclopirox inhibits the mammalian target of rapamycin (mTOR) protein [26], thereby potentially activating autophagy (including in immune cells), which may contribute to host defense against pathogens [27]. These properties offer a distinct advantage in combating resistant bacteria, as they do not rely solely on a direct drug-specific effect but rather engage multiple endogenous host mechanisms. Consequently, ciclopirox may provide additional *in vivo* benefits beyond its direct antibacterial effect and iron chelation, particularly in bacterial infections accompanied by severe inflammation, such as those caused by the pathogens tested in this study [14]. However, the relatively high MBC values observed, particularly for *Y. pestis* (Kim53), suggest that ciclopirox may face pharmacokinetic challenges if utilized as a systemic monotherapy, as limited data from primary phase I paper by Minden et al. [28] reported for oral ciclopirox given once daily at ~80 mg/m², PK values of C_{max} ~220 ng/mL, plasma AUC ~750 ng h/mL and rapid elimination t_{1/2} of ~2.7 h.

To address these hurdles, the clinical presentation of pneumonic plague and respiratory tularemia provides a strong rationale for investigating localized, inhalation-based delivery. Such aerosolized administration could potentially achieve high local concentrations within the pulmonary environment that significantly exceed systemic plasma thresholds, thereby bypassing the limitations associated with systemic administration. Additionally, the use of fosciciclopirox, the phosphoryl-oxymethyl-ester prodrug of ciclopirox-presents a viable solution for systemic delivery, as its maximum tolerated dose (MTD) in mice and humans is much higher than that of ciclopirox. Upon intravenous administration, fosciciclopirox undergoes rapid and complete conversion to the active metabolite, ciclopirox.

Given the observed MIC and MBC ranges, ciclopirox may be most strategically applied as a synergistic adjuvant or potentiator within a multi-drug framework. By disrupting iron and other polyvalent metal ions homeostasis and compromising bacterial metabolic pathways, ciclopirox may sensitize these pathogens to frontline antibiotics, or other repurposed drugs. This potentiation could potentially lower the required therapeutic doses of traditional agents and mitigate the emergence of further resistance. Furthermore, the clinical presentation of pneumonic plague and respiratory tularemia provides a rationale for investigating alternative delivery routes. Aerosolized or inhalation-based administration of ciclopirox could potentially achieve high local concentrations within the pulmonary environment, reaching levels that significantly exceed systemic plasma thresholds. Such localized delivery strategies may bypass the pharmacokinetic limitations associated with systemic administration, offering a more feasible therapeutic pathway for treating primary respiratory infections caused by these Tier-1 pathogens. Future studies focusing on *in vivo* combination efficacy and pulmonary distribution are warranted to further define the clinical potential of ciclopirox in these scenarios.

Despite the antibacterial activity observed in this study, several limitations must be acknowledged. First, while we have validated the efficacy of ciclopirox against both attenuated and virulent strains, our experiments were conducted *in vitro*. This does not

necessarily reflect the complex host–pathogen interactions or host-mediated iron sequestration that may influence drug performance. While the *in vitro* results provide a critical proof-of-concept, the absence of *in vivo* validation means that key parameters—including systemic toxicity, pharmacokinetics, and the ability of the drug to reach therapeutic concentrations within infected tissues—remain to be determined.

In conclusion, the results presented in this study demonstrate as proof of concept that ciclopirox has an antibacterial effect against *Y. pestis* and *F. tularensis*, including virulent strains and avirulent strains possessing non-WT-reduced susceptibility to ciprofloxacin and doxycycline, through a mechanism of action involving primarily iron chelation. These findings indicate that ciclopirox could potentially serve as a combined antibacterial treatment or adjuvant for infections caused by first-choice drugs resistant *Y. pestis* and *F. tularensis* strains.

4. Materials and Methods

The primary objective of this study was to identify and evaluate potential therapeutic options against *Yersinia pestis* and *Francisella tularensis* by assessing the *in vitro* efficacy of ciclopirox. We explicitly state that no experimental procedures were performed with the intent to enhance the pathogenicity, transmissibility, or resistance of the strains used in this study. All methodologies were strictly limited to standard antimicrobial susceptibility testing and the evaluation of drug efficacy to address the public health need for novel treatment strategies against these agents.

Tested strains—all experiments with virulent strains *Yersinia pestis*, Kimberley53 (termed in short Kim53) and *Francisella tularensis* Schu S4 were conducted in a biosafety level 3 (BSL3) facility in accordance with the biosafety guidelines of the Israel Institute for Biological Research (IIBR). Three avirulent derivatives of Kim53 were used: Kim53Δ70Δ10 (Kim53pPCP1[−]pCD1[−]), Kim53Δ70Δpgm (Kim53pCD1[−]pgm[−]) and Kim53Δ10Δpgm (Kim53pPCP1[−]pgm[−]). All derivatives kept the wild-type susceptibility to doxycycline and ciprofloxacin of the parental Kim53 virulent strain. Selected mutants of the plasmid-cured Kim53Δ70Δ10 with reduced susceptibility to ciprofloxacin (MIC within CLSI “non-susceptible” category) were YP-1, YP-2, YP-3, YP4, YP-5, YP6, and YP-7 [29], and mutants with MICs within the CLSI resistant category to doxycycline were YP-8 and YP-9 [30]. *Francisella tularensis* LVS (Live Vaccine Strain), is an avirulent tularemia strain. LVS was similarly mutagenized to achieve derivatives LVS-2 and LVS-3 with MICs categorizing them as nonsusceptible to ciprofloxacin, and LVS-4 with reduced susceptibility to doxycycline (yet categorized as susceptible according to CLSI guidelines for potential agents of bioterrorism) [16]. The isolation was performed in compliance with Israeli law for working with select agents, approved by the Institutional “Recombinant DNA Experimental Usage Committee” and performed according to specific Institutional “Biosafety committee” guidelines for containment and working procedures. Experiments using the virulent strain were performed using biosafety level 3 (BSL-3) containment and procedures. Experiments with the non-virulent strains were performed using biosafety level 2 (BSL-2) containment and procedures.

Reagents and drugs—Ciclopirox olamine (Sigma-Aldrich Rehovot Israel 7670603, Cat. , 1134030), Doxycycline hyclate, Ciprofloxacin (Ciprofloxacin IV Altan Pharmaceuticals S.A. 28230 Las Rozas, Madrid, Spain 2 mg/mL), FeSO₄ (Sigma-Aldrich Rehovot Israel 7670603, Cat. 935689), PBS (Dulbecco’s Phosphate Buffered Saline, Biological Industries Kibbutz Beit HAEMEK 25115 Israel., Cat. 02-023-1A).

Growth Media—Modified Cystine Heart Agar (mCHA) [17] plates were used for isolation and for cfu counts of *F. tularensis* (Schu S4 and LVS). Hematin liquid Mueller Hinton (cations adjusted) IsoVitaleX (HLMHI) broth was used for culturing and antibacterial susceptibility testing of *F. tularensis* strains. The medium is the reference medium for

microdilution tests for *F. tularensis* (CLSI, M45) [16], with addition of 6 μ M Hematin (Sigma-Aldrich, 3281), which enhances the growth of *F. tularensis* in the liquid culture without affecting the susceptibility to ciprofloxacin or doxycycline. Brain Heart Infusion Agar (BHIA, BD Difco 241830) plates were used for isolation and cfu counts of *Y. pestis*. Liquid Medium-Mueller-Hinton-cation-adjusted broth (CAMHB) (BD Difco, 212322) is the reference medium for determining commonly isolated bacterial susceptibility (CLSI, M07) [17]. CAMHB was used for *Y. pestis* in susceptibility tests (CLSI, M45) [16] and for exposure assays with increasing concentrations of ciprofloxacin or ciprofloxacin. Liquid Brain Heart Infusion (L-BHI, Difco, 237500) broth was used for culturing *Y. pestis*. Tryptic Soy Broth (Becton, Dickinson Company 1 Becton Drive Franklin Lakes, NJ 07417-1880 USA Cat. 236950) + 0.1 g/100 mL Cysteine (Sigma-Aldrich, C6852) (TSB-C) for culturing LVS. This medium was used for monitoring MIC changes under selective pressure from exposure of LVS to increasing concentrations of ciprofloxacin or ciprofloxacin.

Microdilution MIC (Minimal Inhibitory Concentration) assay—The MIC was determined using a microdilution assay to establish the minimal concentration of the tested compound that inhibits the growth of a standard bacterial inoculum (CLSI, M07 and M45) [16]. The test was performed by preparing colony suspensions at a concentration of $1-2 \times 10^8$ cfu/mL by adjusting suspensions turbidity, measured with a Novaspec plus spectrophotometer (Amersham Scientific) to O.D._{660nm} = 0.075 for *F. tularensis* in HLMHI medium, or O.D._{660nm} = 0.31–0.34 for *Y. pestis* in CAMHB medium. The calibrated suspension was diluted 1:100 in the appropriate medium and inoculated (50 μ L) into flat-bottom 96-well microplates (TPP, Cat. No. 92096) containing serial dilutions of the tested antibacterial compounds (50 μ L), resulting in a final well volume of 100 μ L and an initial bacterial concentration of approximately 5×10^4 – 1×10^5 cfu/well (or 5×10^5 – 1×10^6 cfu/mL) in the respective test medium. All tests were done in duplicates and repeated in at least three independent days. Dilutions for viable cfu counts were performed to verify the inoculum concentration on the appropriate agar medium for each bacterium. Turbidity monitoring was carried out using a Tecan Infinite plate reader (Tecan Austria GmbH, Untersbergstr. 1A, A-5082 Grödig, Austria), with hourly measurements of at O.D.₆₃₀ for an incubation period of 24–48 h at 28 °C for *Y. pestis* or 48–72 h at 37 °C for *F. tularensis*. At the end of incubation, the threshold for growth/inhibition in the wells was defined as 10% relative to the growth control wells without the tested compound. The MIC was established both by the turbidity (O.D.₆₃₀ < 10% of growth control) and visually (un-aided eye). MIC values were determined after incubation of 24 h for *Y. pestis* and 48 h for *F. tularensis*, in accordance with CLSI M45 guidelines. Media and antibiotic dilutions were verified using quality control strain *E. coli* ATCC 25922.

Minimum Bactericidal Concentration (MBC)—characterization experiments to determine the MBC in the presence of ciprofloxacin were conducted in triplicates using the same conditions used for the microdilution MIC tests.

The MIC was established by monitoring turbidity both visually (un-aided eye) and using a plate reader (O.D. $\leq$ 10% of growth control). At the time of MIC determination, all wells from the MIC value and higher (showing no visible growth) were sampled and serially diluted in PBS and plated on agar plates with the appropriate medium for CFU counts. The MBC was defined as the minimal concentration at which a reduction of 99.9% (<0.1% survival) in the number of surviving bacteria occurs relative to cfu in time zero. The MIC and MBC values were used to assess the bacterial susceptibility and to characterize whether the activity of ciprofloxacin is bactericidal (MIC/MBC ratio $\leq$ 4) or bacteriostatic (MIC/MBC ratio $\geq$ 8).

Time-Kill Curve (TKC)—Characterization of the TKCs for ciprofloxacin was determined using the microdilution test method, in which the MIC for *Y. pestis* and *F. tularensis* was assessed at 24 and 48 hours respectively by monitoring turbidity visually and with a plate

reader, accompanied by replicate test plates incubated under the appropriate temperature conditions. At predefined time points, duplicate wells were sampled, pooled for serial tenfold dilutions in PBS and plated on suitable agar plates, for CFU enumeration and quantification of surviving percentage of bacterial counts relative to the initial inoculum at time zero.

Iron Effect—The effect of iron chelation on bacterial sensitivity was determined under the same MIC test conditions, using CAMHB medium for *Y. pestis* and HLMHI medium for *F. tularensis*. The tested bacteria were suspended to a turbidity of O.D.₆₆₀ = 0.31–0.34 for *Y. pestis* and O.D.₆₆₀ = 0.073–0.078 for *F. tularensis*, diluted 1:100 in the appropriate medium and inoculated into wells containing medium with various concentrations of FeSO₄ solution at each tested ciprofloxacin concentration. The *Y. pestis* (Kim53 and plasmids/*pgm*-cured derivatives) test plates were incubated at 28 °C and the *F. tularensis* (LVS Schu S4 and) plates at 37 °C, in a Tecan infinite plate reader, with continuous hourly monitoring of culture turbidity at O.D.₆₃₀ nm for 24–72 h, to assess the effect of iron presence on bacterial sensitivity (MIC values) to ciprofloxacin.

Selective pressure—for preliminary screening of resistance isolates selection was done by plating *Y. pestis* (Kim53) on BHIA at 28 °C for 48 h and *F. tularensis* LVS on mCHA at 37 °C for 72 h. Colonies were suspended in CAMHB (Kim53 Δ 70 Δ 10) or TSB-C (LVS) to 1 OD_{660nm}. The following two drug series were prepared: ciprofloxacin at 0.5 $\times$, 1 $\times$, 2 $\times$, and 4 $\times$ MICs; ciprofloxacin at 0.25 $\times$, 0.5 $\times$, 1 $\times$, and 2 $\times$ MICs. Bacterial suspensions were diluted to 0.001 OD_{660nm} in 5 mL of indicated medium and incubated shaking at 180 rpm for 48 h at 28 °C (Kim53 Δ 70 Δ 10) or 72 h at 37 °C (LVS). After incubation, turbidity was measured. Culture with highest concentration was used to inoculate fresh medium with a new dilution series, where the lowest concentration matched 0.25 $\times$ the previous highest growth concentration. This selection process was repeated for up to 12 passages or until reduced sensitivity was achieved for both ciprofloxacin and ciprofloxacin. At each cycle passage, cultures were plated (BHIA for Kim53 Δ 70 Δ 10; mCHA for LVS) to verify identity and MICs determination, isolates with reduced MICs were kept for future molecular and omics-based analysis.

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Dual Use Research of Concern (DURC) statement: Current research is limited to the field of antimicrobial drug discovery and clinical microbiology, which is beneficial for identifying repurposed therapeutic alternatives to combat antibiotic-resistant infections and does not pose a threat to public health or national security. Authors acknowledge the dual use potential of the research involving *Yersinia pestis* and *Francisella tularensis* and confirm that all necessary precautions have been taken to prevent potential misuse. As an ethical responsibility, authors strictly adhere to relevant national and international laws about DURC. Authors advocate for responsible deployment, ethical considerations, regulatory compliance, and transparent reporting to mitigate misuse risks and foster beneficial outcomes.

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