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Research article

Carbapenem Resistance is Associated with Biofilm Formation in Clinical Isolates of *Klebsiella pneumoniae*

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ABSTRACT

Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) and biofilm-producing nosocomial pathogenic bacteria may complicate treatment and infection control. The relationship between carbapenem resistance and biofilm formation remains poorly characterized. Susceptibility to imipenem, doripenem, ertapenem, amikacin, and levofloxacin was assessed using the Kirby-Bauer disk diffusion assay and CLSI M100 breakpoints for ninety-five non-duplicate clinical isolates of *K. pneumoniae*. The crystal-violet microtiter assay, which measured the optical density at 570 nm and was categorized based on Stepanovic criteria, was used to measure the ability of the isolates to produce biofilm in vitro. Based on the isolates' carbapenem resistance results, the isolates were classified as carbapenem-resistant (CR), carbapenem-intermediate (CIN), and carbapenem-susceptible (CS). The susceptibility rates were 74.0%, 68.8%, 63.5%, 46.0%, 73.1%, and 68.4% for imipenem, doripenem, meropenem, ertapenem, amikacin, and levofloxacin, respectively. The results showed that 42.1% (40/95) were resistant to a carbapenem antibiotic, while 17.4% (16/92) were multidrug-resistant. When the biofilm product was assessed, 35.8% of the isolates were moderate biofilm producers, 44.2% were strong biofilm producers, and 20.0% were weak biofilm producers. The OD570 of biofilm formation was significantly higher in CR isolates than in CS isolates (0.59 vs. 0.424; Mann-Whitney U=976.5, p= 0.03). Resistance to carbapenems was significantly correlated with biofilm formation (Spearman ρ = 0.224, p = 0.028). Isolates with strong biofilm formation were more frequent in CR-KP than in non-CR-KP (55.0% vs. 36.36%), but the distribution was not significant (χ^2 =3.16, p =0.206). Carbapenem resistance was common and associated with a moderate increase in biofilm production. This study recommends continued surveillance, and large genotypic studies are warranted.

Keywords: Antibacterial susceptibility; Biofilm formation; Carbapenem resistance; Kirby-Bauer disk diffusion; *Klebsiella pneumoniae*; multidrug resistance.

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1. INTRODUCTION

Klebsiella pneumoniae is one of the most clinically important species of the Enterobacteriaceae and is responsible for hospital-acquired infections, pneumonia, wound infections, and urinary tract infections [1]. The carbapenem-resistant Enterobacterales, including *K. pneumoniae*, are designated by the, World Health Organization as priority pathogens on the 2024 list, as a critical-priority bacterial group with limited treatment options and associated with mortality of these infections [2]. This pathogen persists in the hospital environment and is present in indwelling devices through the production of biofilm, a structured, biomass-encased community that provides a barrier, reduces antibiotic penetration, reduces the effectiveness of phagocytosis, and complicates eradication, even when planktonic susceptibility testing indicates that an isolate must

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respond to therapy [1]. In addition to its ability to form biofilms, this pathogen has acquired resistance determinants such as carbapenemase production, porin loss, and efflux-pump overexpression [3]. All of these make *K. pneumoniae* one of the most common hospital-acquired pathogens. Many clinical collections assess the connection between antimicrobial resistance, specifically carbapenem resistance, and ability of bacteria to form biofilm, but results are inconsistent: some studies find that there is a positive correlation between carbapenem resistance and biofilm formation [4] while other investigations, such as one large single-center US study, find the opposite that isolates that are resistant to carbapenems are significantly less likely to form strong biofilm indicating a potential fitness trade-off between resistance and adhesion-related virulence factors such as type 3 fimbriae [5,6].

The relationship between the ability of *K. pneumoniae* to produce biofilm and resistance to carbapenem groups seems to depend on the isolate collection, patient population, and local resistance mechanisms [7]. Thus, it would be more useful to show this empirically in a clinical dataset rather than assume a universal direction of the effect. Here, we analyze disk-diffusion susceptibility data and crystal violet biofilm assay data from 95 *K. pneumoniae* clinical isolates to (i) identify the current antimicrobial susceptibility profile with respect to carbapenems, one aminoglycoside, and one fluoroquinolone, and (ii) determine whether the biofilm formation ability is different for carbapenem-resistant and carbapenem-susceptible isolates.

1. MATERIALS AND METHODS

1.1. Bacterial Isolates

The study included 95 non-duplicated clinical isolates of *K. pneumoniae*. The isolates were obtained from three main hospitals in Baghdad and from various medical centers in Baghdad and Babylon, Iraq. The isolates were obtained from urine samples of patients with UTIs. The clinical isolates were re-identified by culturing on various media, including MacConkey and eosin methylene blue agar. Biochemical tests performed included oxidase, catalase, indole, citrate utilization, urease, and motility tests. The Vitek II system was used to confirm the species of the isolated bacteria. The *K. pneumoniae* isolates were stored short-term by culturing on nutrient agar slants and keeping them at 4 °C. The isolates were also stored long-term by culturing in 20% nutrient broth and keeping them at -20 °C for a year [8,9].

1.2. Antibiotic Susceptibility Testing

The Kirby-Bauer disk diffusion method on Mueller-Hinton agar was used to determine the susceptibility of 95 clinical isolates of *K. pneumoniae*. The isolates were cultured on Mueller-Hinton broth (HiMedia, India) overnight at 37 ºC. The bacterial cells were washed three times with sterile normal saline at 10000 rpm (High-Speed Centrifuges, Lab Equipment, USA). The turbidity of the bacterial suspension was adjusted to 0.1 at 600 nm (spectrophotometer, Shimadzu Corporation, Japan). The bacterial suspension was spread onto MH Agar (Himedia, India). The antibiotic disks of imipenem (IMP, 10 µg/mL), doripenem (DOR,10 µg/mL), meropenem (MEM, 10 µg/mL), ertapenem (ETP, 10 µg/mL), amikacin (AMK, 30 µg/mL), and levofloxacin (LEV, 5 µg/mL) were added to the agar surface. The plates were incubated overnight at 37 ºC.

The diameters of inhibition zones were measured in millimeters (mm). The results were interpreted as susceptible (S), intermediate (I), and resistant (R) based on the Clinical and Laboratory Standards Institute (CLSI) breakpoints for the *K. pneumoniae* (Enterobacterales): IMP, DOR, and MEM (R ≤ 19 mm, S ≥ 23 mm, I= 20-22); ETP (R ≤ 18 mm, S ≥ 22, I= 19-21); AMK (R ≤ 14 mm, S ≥ 17, I= 15-16); and LEV (R ≤ 13 mm, S ≥ 17, I= 14-16) [10].

Certain results were reported simply as either S or R (with no measurable zone or complete inhibition) and were, therefore, recorded with the respective category rather than additional values; these were acknowledged accordingly. Isolates that were resistant to one or more studied carbapenems received a designation of carbapenem-resistant *K. pneumoniae* (CR-KP); those that were susceptible to all tested carbapenems were labeled as carbapenem-susceptible (CS), while strains with intermediate values but no direct resistance were recorded as carbapenem intermediate. Multidrug resistance (MDR) was defined in accordance with the interim recommendations by Magiorakos et al. as the resistance of the strain (either I or R) to at least one agent in at least three of the three antimicrobial classes investigated (carbapenems, fluoroquinolones, or aminoglycosides) whereby isolates with results in all three categories were subject to evaluation (total of 95) [11].

1.3. Biofilm Formation Assay

The crystal violet microtiter plate assay with OD read at 570 nm was used to evaluate the ability of clinical isolates of *K. pneumoniae* to produce biofilm in vitro. In the experiment, the mean of three replicate readings was used as each isolate's biofilm. In this method, 100 µL of trypticase soy broth (TSB, Hi Media, India) supplemented with 0.5% glucose was added to each well of a 96-well flat-bottom polystyrene microtiter plate. Five microliters of bacterial suspension (bacterial isolates were grown in TSB overnight; the bacterial cells were washed three times with PBS, and the final turbidity of the bacterial suspension was adjusted to 0.1 at 600 nm) were added to each well. The plates were incubated overnight at 37°C. The wells were washed gently with sterile normal saline three times, and the attached biomass (biofilm matrix) was then dried and fixed by incubation in an oven at 60°C for an hour. Hundred microliters of crystal violet were added to each well. The plates were incubated at room temperature for 15 min, then the wells were washed three times with PBS. The plates were dried, and 100 µL of absolute ethanol (Fluke, UK) was added. The plates were incubated for 30 min at room temperature, and the OD at 570 was measured (UV-1900i, Shimadzu Corporation, Japan) [12].

The control cutoff density (ODc) specific to the study is calculated. The Stepanović approach was used to calculate the cutoff value and to divide the isolates into four categories. The categories are as follows: non-producers (OD ≤ ODc), weak (ODc < OD ≤ 2×ODc), moderate (2×ODc < OD ≤ 4×ODc), and strong (OD > 4×ODc) [13]. Since the cutoff value is literature-based, its absolute values have to be considered with caution; however, quantitative values (OD570 data) along with comparative studies are essential

1.4. Data and Statistical Analysis

In the current study, counts and percentages of every variable have been used to present categorical variables (distribution of the susceptible/Intermediate/resistant strains and biofilm types). Biofilm OD570 is given as mean ± standard deviation and median (range). The difference in biofilm OD between isolates resistant and susceptible to carbapenems was analyzed using the Mann-Whitney U test due to the non-normal, right-skewed distribution typical of optical density data. We compared the

distribution of biofilm type (weak/moderate/strong) in carbapenem-resistant and non-resistant strains using the chi-square test. To measure the association between the number of carbapenems to which the isolates are resistant (0-4) and biofilm OD570, the Spearman rank correlation coefficient was calculated. A two-sided p-value < 0.05 was considered statistically significant. Analyses were performed in Python 3 (SciPy).

2. RESULTS

2.1. Antibacterial Susceptibility Profile

Using the Kirby-Bauer disk diffusion method, the susceptibility rates for 95 *K. pneumoniae* isolates showed the highest susceptibility to imipenem (74%) and amikacin (73.1%) with intermediate susceptibility to doripenem (68.8%) and levofloxacin (68.4%), followed by meropenem (63.5%), and the lowest susceptibility was found with ertapenem. The resistance rates varied from 12.9% (amikacin) to 34.0% (ertapenem, the tested subset). The full S/I/R distributions are illustrated in Table 1 and Fig. 1.

Table 1. Antimicrobial susceptibility of 95 clinical *K. pneumoniae* isolates by Kirby-Bauer disk diffusion, interpreted per CLSI M100 (34th ed.) breakpoints for Enterobacterales. n tested excludes isolates with a not-determined or not-tested result for that agent.

Antimicrobial agent	n tested	S (%)	I (%)	R (%)
Imipenem	95	74.0	7.3	18.8
Doripenem	95	68.8	8.3	22.9
Meropenem	95	63.5	10.4	26.0
Ertapenem	95	46.0	20.0	34.0
Amikacin	95	73.1	14.0	12.9
Levofloxacin	95	68.4	7.4	24.2

2.2. Carbapenem Resistance and Multidrug Resistance

After combining all results concerning the four carbapenem studies, it was found that 40 isolates belonging to the total of 95 studied (42.1%) were classified as CR-KP; out of those, 18 isolates (18.9%) had intermediate results towards at least one carbapenem and 37 isolates (38.9%) were classified as CS-KP. Results could be obtained for 92 isolates regarding all three antimicrobial categories used (carbapenems, amikacin and levofloxacin), that is the classification of multidrug resistant strains was only for 16 isolates (17.4%).

2.3. Biofilm Formation

All isolates in the study were detected by the crystal-violet assay to form biofilm. In accordance with the Stepanović criteria for biofilm classification, 42 isolates (44.2%) were classified as high biofilm producers, 34 isolates (35.8%) were classified as moderate producers, and 19 isolates (20.0%) were classified as low biofilm producers. The average OD570 of the population was 0.517 ± 0.305 (median of 0.452), suggesting great variation in the biofilm-forming ability of different strains.

2.4. Carbapenem Resistance and Biofilm Formation

The average of biofilm OD570 levels increased through the spectrum of carbapenem tolerance: 0.424 for carbapenem-sensitive isolates (n = 37), 0.541 for carbapenem-intermediate isolates (n = 18), and 0.590 for carbapenem-resistant isolates (n = 40). A significant difference was found between resistant and sensitive isolates (Mann-Whitney U = 976.5, p = 0.030). Biofilm formation was numerically more prominent in CR-KP isolates (22 out of 40, or 55%) compared to non-CR-KP ones (20 out of

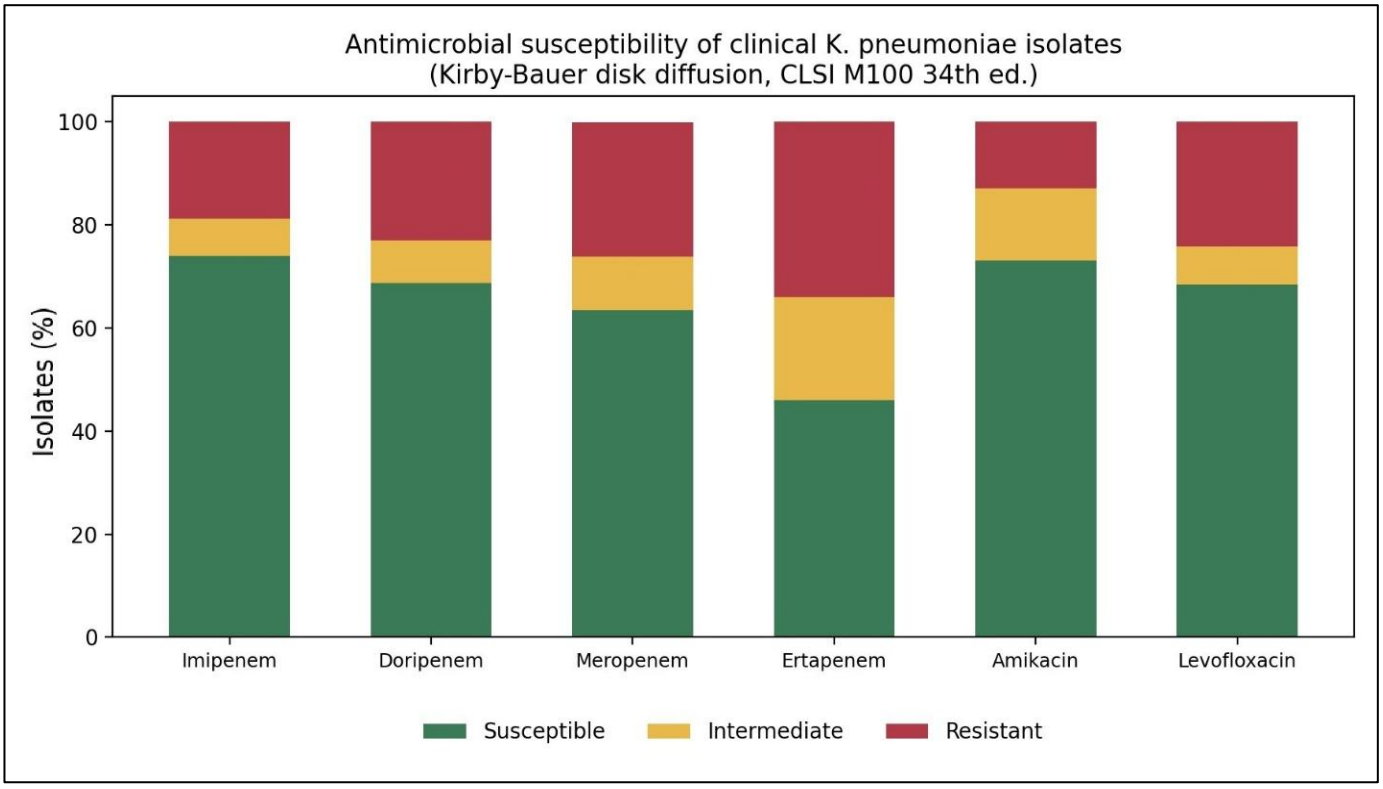

Fig. 1. The rate of Susceptibility (S), intermediate (I), and resistance (R) for six antimicrobial agents among 95 clinical isolates of *K. pneumoniae*. Numbers within the bars indicate the percentage of tested isolates in each category.

55, or 36.4%); however, the entire three-category distribution (weak/moderate/strong) did not reach statistical significance ($\chi 2 = 3.16$, df = 2, p = 0.206). The total number of carbapenems to which the isolate was resistant (0-4) showed a weak but statistically significant correlation with biofilm OD570 (Spearman ρ = 0.224, p = 0.028) (Table 2) (Fig. 2)

Table 2. Biofilm formation in terms of optical density (OD570, crystal-violet assay, mean of triplicate wells) by carbapenem susceptibility category. Mann-Whitney U (CR-KP vs CS) = 976.5, p = 0.030; chi-square for weak/moderate/strong distribution, CR-KP vs non-CR-KP: $\chi^2 = 3.16$, p = 0.206.

Carbapenem status	n	Mean OD570 ± SD	Median OD570	Strong biofilm producers, n (%)
Carbapenem-susceptible (CS)	37	0.424 ± 0.238	0.354	—
Carbapenem-intermediate	18	0.541 ± 0.279	0.464	—
Carbapenem-resistant (CR-KP)	40	0.590 ± 0.336	0.523	22 (55%)
Non-CR-KP (CS + intermediate)	55	—	—	20 (36.36%)

3. DISCUSSION

Antibiotic resistance, especially to carbapenems, poses a serious challenge for physicians, as these antibiotics represent the last line of defense against bacterial infections. Disrupting this defense line raises a red flag for global public health, signaling a serious threat worldwide [14]. The current study involved ninety-five isolates of *K. pneumoniae* from urinary tract infections. The study showed that carbapenem resistance remained common: 42.1% of the isolates were resistant to at least one of the four carbapenems tested by the Kirby-Bauer disk diffusion method. The study also showed that susceptibility testing with ertapenem had the lowest susceptibility rate, confirming its recognized role as the most sensitive indicator carbapenem for determining emerging carbapenem-mediated resistance in *K. pneumoniae*, because its zone diameter is affected earliest by low-level resistance mechanisms. The study showed that the percentage of susceptibility for amikacin and imipenem was relatively similar, indicating these antibiotics could constantly show good in vitro activity in the studied cohort, but clinical decisions should be based on isolate-specific results rather than on overall rates.

The important conclusion of this study is that a statistically significant relationship exists between carbapenem resistance and biofilm formation; in particular, there is a much higher average biofilm OD570 for carbapenem-resistant strains than for susceptible strains (0.590 vs 0.424, p = 0.030). The study also showed that the number of different carbapenem agents against which a strain is resistant is only weakly correlated with biofilm OD (ρ = 0.224, p = 0.028). The positive direction of the relationship found is supported by various clinical studies. Rahdar et al. (2019) showed a clearly significant relationship between the ability to form biofilm and carbapenem resistance among 160 *K. pneumoniae* isolates [4]. pneumoniae isolates, the same study showed that the most of the resistant strains have been shown to belong to the group of strong or moderate biofilm producers. Similarly, studies of a collection of *K. pneumoniae* strains associated with devices from critically ill patients reported a positive correlation between the ability to form biofilm and carbapenem resistance [15]. In addition, other single-center studies have also reported high proportions of biofilm-positive strains among antibiotic-resistant *K. pneumoniae* isolates.

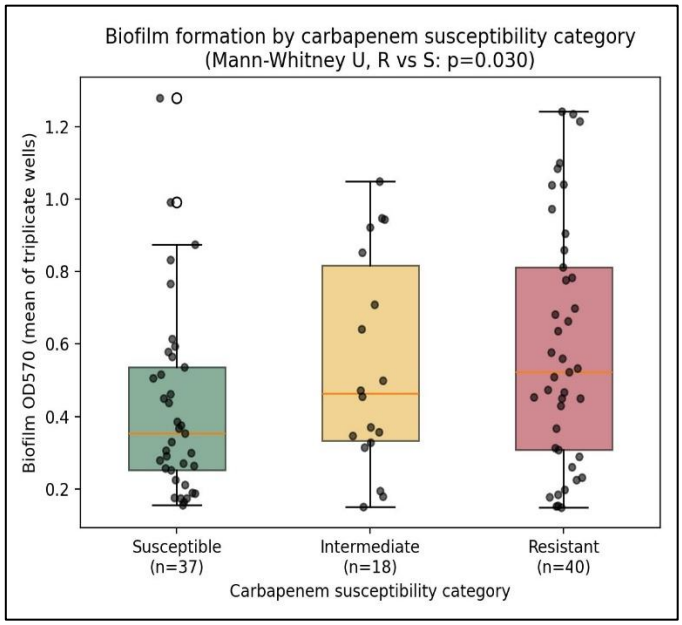

Fig. 2. Distribution of biofilm by carbapenem susceptibility category (susceptible, intermediate, resistant). Boxes show median and interquartile range; points show individual isolates.

This finding is not common. Cusumano et al. (2019) reported the opposite pattern of what we found in the current study. They found that CRKP were 91% less likely to produce strong biofilm than carbapenem-susceptible *K. pneumoniae* (CSKP) isolates, and MDR isolates produced weak biofilm [5]. Similarly, Fang et al. (2021) found that carbapenem-resistant isolates produced weak biofilm compared with carbapenem-susceptible isolates, which showed a slightly higher capacity for biofilm production. This was attributed to reduced expression of the mrkH regulator of type 3 fimbriae in carbapenem-resistant isolates [6]. A possible explanation for the disparate results is through the idea of competing fitness: gaining and retaining the genes behind carbapenemase production and the changes to the porins or efflux pump may require a metabolic expense making certain lineages less efficient with their investment of virulence factors related to adhesion and the extracellular matrix, while for others, particularly those where resistance and formation of biofilm factors are regulated together or are found on the same mobile genetic unit, the association may work the other way around [16, 17]. Thus, the present observations conform to this interpretation. Nonetheless, strain-level genotyping (such as regarding the types of carbapenemase genes, capsular genes or fimbrial genes lost) will be needed to identify the mechanism linking resistance against carbapenems and biofilm formation in this case [18,19].

A clinical viewpoint suggests that biofilm-forming capacity was, if anything, greater than that of the carbapenem-resistant isolates (CRKP) in this study. This could imply that carbapenem-resistant isolates may not only be limited in their treatment choices due to their resistance but may also be able to establish biofilm infections using medical devices or in chronic infections, which might be difficult to eliminate even if an appropriate antimicrobial was chosen, since biofilm bacteria are known to be

highly resistant to drugs [20]. The need for source control (e.g., removal of catheter or device) along with appropriate antimicrobial should be kept in mind during the management of infections caused by *K. pneumoniae* that produce biofilms and are resistant to carbapenems [21].

In the present study, two limitations were specified: first, genotyping of carbapenemase was not done, which made it impossible to perform mechanistic interpretation regarding the association of biofilm with resistance. Second, clinical and epidemiological metadata (site of specimen collection, patient outcomes and relation to medical devices) were not available to correlate with the microbiological data.

4. CONCLUSION

The data indicate that carbapenem resistance persists in *K. pneumoniae* isolates, and all isolates tested were capable of biofilm formation. Carbapenem-resistant isolates showed a slightly higher tendency for biofilm formation than carbapenem-susceptible strains. This adds to the body of evidence linking carbapenem resistance and biofilm formation in bacteria. Based on this finding, we suggest including biofilm testing in routine testing procedures to optimize the management of infections caused by the mentioned pathogen.

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Conflict of interest

The authors declare no conflicts of interest.

Ethical Approval

The current study was conducted following approval from the Ministry of Health, Baghdad, Iraq (Reference number 1102, Date: 2, 10, 2025).

Author contributions

Jabuk SIA. Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing – original draft.

Heydarlou MM. Methodology, Investigation, Data curation, Formal analysis, Writing – review & editing.

Shawal M. Methodology, Investigation, Validation, Writing – review & editing.

Rehman AU. Supervision, Project administration, Resources, Writing – review & editing.

All authors reviewed and approved the final manuscript and agreed to be accountable for all aspects of the work.

AI declaration

During the preparation of this work, the authors used Claude AI and in order to refine the academic tone and improve the readability of the manuscript. After using this tool/service, the authors reviewed and edited the content as needed and took full responsibility for the content of the publication.

Data availability

Data will be made available on request.

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