integrity Research Journals

Journal of Bioscience and Biotechnology Discovery
Volume 10(2), pages 37-43, June 2025
Article Number: F65D6CE12
ISSN: 2536-7064
https://doi.org/10.31248/JBBD2025.231
https://integrityresjournals.org/journal/JBBD

Full Length Research

Plasmid curing of bacteria isolated from disinfectants used in four hospitals

Kyahar, I. F.* and Olorunfemi, P. O.

Department of Pharmaceutical Microbiology and Biotechnology, University of Jos, Nigeria.

*Corresponding author. Email: kyaharfriday@yahoo.com; Tel: +234-8034531378.

Received 13th March 2025; Accepted 24th May 2025

ABSTRACT: The presence of resistant plasmids (R-plasmids) in microorganisms allows them to evade antibiotics, complicating infection treatment. Disinfectants, though essential in infection control, can become contaminated, contributing to antimicrobial resistance in hospital environments. This study investigated plasmid-mediated antibiotic resistance in bacteria isolated from used disinfectants across four hospitals by evaluating their susceptibility before and after plasmid curing. A total of 100 disinfectant samples (both used and unused diluted forms) were collected, with 21% found contaminated, 86% of which were Gram-negative bacteria. The most frequent isolates included *Pseudomonas aeruginosa* (24%), *Klebsiella pneumoniae* (19%), *Escherichia coli* (14%), *Proteus vulgaris* (14%), *Salmonella typhi* (14%), and *Staphylococcus aureus* (14%). Antibiotic susceptibility tests showed that 81% of isolates were sensitive to all 17 antibiotics tested, while 19% exhibited multidrug resistance, particularly *Pseudomonas*, *Klebsiella*, *Salmonella*, and *E. coli*. Ampicillin showed the highest resistance, while ciprofloxacin and levofloxacin retained effectiveness. Plasmid curing revealed partial plasmid-mediated resistance in key bacteria. Before curing, *Salmonella typhi*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *E. coli* resisted 15, 9, 8, and 7 antibiotics, respectively (100%). After curing, resistance reduced to 67% in *Salmonella*, 78% in *Klebsiella*, 88% in *Pseudomonas*, and 57% in *E. coli*, indicating loss of resistance to specific antibiotics. This suggests that resistance to erythromycin, amoxicillin, norfloxacin, and septrin in *Salmonella*; norfloxacin and septrin in *Klebsiella*; septrin in *Pseudomonas*; and norfloxacin, chloramphenicol, and septrin in *E. coli* was plasmid mediated.

Keywords: Antibacterial susceptibility, bacterial resistance, cross-resistance, disinfectant bacteria isolates, infection control protocols.

INTRODUCTION

Plasmids are independent, circular, self-replicating extra-chromosomal DNA elements with characteristic copy numbers within the host. These plasmids encode various properties, including resistance to antibiotics and heavy metals, degradation of hydrocarbons, and the synthesis of bacteriocins and antibiotics. Plasmid-mediated antibiotic resistance can be easily transferred from one bacterium to another through transformation, conjugation, or mobilisation (Patwardhan *et al.,* 2018). Resistance to multiple antibiotics encoded by plasmids has increasingly been recognised as a major challenge in treating infections. In addition to antibiotic resistance, some bacterial plasmids also confer pathogenicity to the host cell (Patwardhan *et al.,* 2018).

Disinfectants are chemical substances intended to reduce the microbial load or eliminate pathogens from surfaces, equipment, and materials within various environments, particularly healthcare settings. Their primary role is to minimise the risk of infection transmission by killing or inhibiting the growth of harmful microorganisms (Russell, 1997). However, improper use of disinfectants, such as incorrect concentration, unsuitable selection for specific pathogens, or inconsistent application, can result in the survival of resistant bacterial strains within disinfectant solutions. This presents a potential risk for cross-resistance between disinfectants and antibiotics, making the treatment of infections more challenging.

The antibacterial susceptibility of bacterial contaminants

found in disinfectants has not been widely studied, but emerging evidence suggests that certain microorganisms can develop resistance to disinfectants over time. Misuse of disinfectants, such as improper concentration, incorrect selection, or inconsistent application, can foster the survival of resistant strains. These resistant bacteria may not only survive in the disinfectant but may also demonstrate cross-resistance to antibiotics, making them harder to treat with conventional medications (Ayliffe *et al.,* 1992).

Hospitals are environments where multiple disinfectants are used for various cleaning purposes, and they often harbour a wide range of bacterial species. These bacteria, when they become resistant to disinfectants, can act as reservoirs of resistance, potentially leading to the spread of resistant organisms throughout the hospital and among patients. Thus, understanding the antibacterial susceptibility of these contaminants is essential to improving infection control protocols (Gilbert and McBain, 2003).

The emergence of disinfectant-resistant bacteria is a growing concern that complicates efforts to control infections in hospitals and other healthcare facilities. These resistant strains are more difficult to eradicate with routine disinfection protocols, leading to the potential spread of nosocomial infections. Moreover, the cross-resistance between disinfectants and antibiotics further complicates treatment options, presenting new challenges in the management of infections. Thus, understanding the factors contributing to disinfectant misuse and resistance development is critical for improving infection control strategies in clinical environments (Gilbert and McBain, 2003).

While much research has focused on antibiotic resistance in clinical isolates, comparatively less attention has been given to disinfectant-resistant bacteria. Nonetheless, studies have begun to reveal that bacterial contaminants can persist in disinfectant solutions, particularly when biocides are used improperly. For instance, pathogens such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Staphylococcus aureus* have been found in used disinfectants in hospital environments, raising alarms about their resilience and adaptive mechanisms (Panigrahi *et al.,* 1996)

MATERIALS AND METHODS

Sources and sample collection

Disinfectant samples (15 from each hospital) were collected from pediatric, surgical and maternity wards, medical laboratories and pharmacy units in four hospitals, namely hospital A, B, C and D in Plateau State. One hundred (100) disinfectant samples of stock solution, unused dilution and used dilution in sterilised 100 ml bottles were collected during morning and afternoon mopping times and packed in a bucket containing ice-cool packs and transported immediately to the research laboratory.

Isolation and characterisation of bacteria procedure

Each of the samples collected was inoculated into 5 ml of brain heart infusion (BHI) broth for enrichment and incubated for 24 hours at 37°C. Colonies of different morphological characteristics were picked from the spread plate culture and streaked on nutrient agar plates and incubated at 37°C for 24 hours. Colonies identifiable as discrete on the nutrient plates were subsequently subcultured on agar slants for identification. The methods used in the identification and characterisation of isolated bacteria included: The colonies were observed under the light microscope after simple Gram staining. Isolation of specific bacteria was done by streaking on selective media. A loopful of inoculum from the 18-hour broth culture was streaked on selective agar and incubated at 37°C for 18 hours. The cultural characteristics of *Escherichia coli* were streaked on MacConkey agar to differentiate coliform (pink colony) from non-coliform (non-lactose fermenters) and checked on Eosin Methylene blue agar. *Pseudomonas aeruginosa* was checked on Cetrimide agar, *Staphylococcus aureus* on mannitol salt agar and *Streptococcus pyogenes* on blood agar. The cultural characteristics were observed and compared to standard references (Thayer *et al*., 1977). They were further subjected to various biochemical tests for confirmation (Cheesbrough, 2002).

Preparation and standardisation of inoculum

The inoculum was standardised by using the Clinical and Laboratory Standards Institute's guideline (CLSI, 2015) as described by Adeshina *et al*. (2010). The twenty-four-hour broth culture of each test organism was standardised by gradually adding normal saline to compare its turbidity to the McFarland standard of 0.5, which is approximately 1.0 x 10^8 cfu/ml. The turbidity of the cell culture was matched with that of the 0.5 McFarland standard by holding the mixture and the standard in front of light against a white background with contrasting black lines through visual comparison with its density by the addition of normal saline.

Antibiotic susceptibility testing

Kirby Bauer disc agar diffusion method and interpreted according to the guidelines of Clinical Laboratory Standards Institute (CLSI, 2015) was used. A 1.0 mL McFarland standard, which was cultured overnight, was used to flood the surface of sterile Mueller Hinton agar plates, the excess drained off, and the plates over-dried. Up to 17 commercially-prepared, fixed concentrations paper antibiotic multi-disks containing: (Ciproflox 10 µg, Norfloxacjn 10 µg, Gentamycin 10 µg, Amoxil 20 µg, Streptomycin 30 µg, Rifampicin 10 µg, Erythromycin 30 µg, Chloramphenicol 30 µg, ampiclox 2 0 µg, levofloxacin

20 µg, taravid 10 µg, Augmentin 30 µg, pefloxacin 10 µg, septrin 30 µg, ciporex 10 µg, Nalidixic acid 20 µg and ampicillin 30 µg were then aseptically placed on the inoculated Mueller Hinton agar plates and allowed to stand for 30 minutes. The plates (prepared in duplicates for each isolate) were then incubated at 37°C and observed after 24 hours incubation period.

The diameters of inhibition zones (ml) were measured using a graduated meter rule, and results were recorded and interpreted following the guidelines of CLSI (2015).

Plasmid curing procedure

The acridine orange method described by Gambo *et al.* (2024) was employed in the curing of the resistant isolates. To a set of 6 test tubes, the first test tube contained 5m1 of double-strength nutrient broth while test tubes numbers 2 to 6 contained 5 ml single of single-strength nutrient broth. 5 ml (0.2%) of the acridine orange solution was added to the first tube and serially diluted (two-fold) to obtain 5 different concentrations (0.1%, 0.05%, 0.025%, 0.0125% & 0.00625%). Tubes 1-5 were then inoculated with 1.0 ml of a standardised overnight culture of the four bacterial isolates considered resistant (*Pseudomonas aeruginosa*, *Salmonella typhi*, *Klebsiella pneumoniae* and *Escherichia coli*). Tube 6 served as a control by not adding to it any acridine orange solution, but was inoculated with the organism. While a tube with medium only was set as a negative control for sterility of the medium and served as a reference for comparing the turbidity of the serially diluted and inoculated single-strength nutrient broth to detect growth by a change in turbidity after an incubation period of 24 hours. After 24 hours of incubation at 37°C, the organisms were freed from the chemical (acridine orange) by subculturing on sterile nutrient agar slants and incubated at 37°C. Antibiotic susceptibility test was done again for the bacterial isolates that showed resistance to more than six (46%) antibiotics prior to the curing, and the changes in resistance patterns were recorded. The bacteria that displayed an increase in the diameter of the zone of inhibition when compared with the values before curing were regarded as bearing their resistance factor in the plasmid.

Testing of bacteria for sensitivity after plasmid curing

The cured bacteria were subjected to a sensitivity test against the same antibiotics to which they were previously resistant to determine if their resistant plasmid was cured as per the disc agar diffusion technique. The cured colonies were inoculated onto the prepared Mueller-Hinton agar plates. Then, antibiotic discs of prior resistance were aseptically introduced into the plates. Ensuring that the disc made appropriate contact with the surface of the agar. This was incubated for 24 hours at 37°C, after which plates were examined.

RESULTS AND DISCUSSION

A total of 100 samples (disinfectants) were collected. The unused and used diluted disinfectant samples were found to be contaminated (21% contamination level). Gram negative bacteria were the predominant contaminants (86%) among which *Pseudomonas aeruginosa* has the highest occurrence (24%), followed by *Klebsiella pneumonia* (19%), *Escherichia coli* (14%), *Proteus vulgaris* (14%), *Salmonella typhi* (14%), and *Staphylococcus aureus* (14%) as shown on Table 1. Tytler *et al.* (2006) reported that Gram-negative bacteria constituted 69% of the microbial contaminants when sampled for similar analysis from three different Northern Nigerian hospitals. Similar findings of the predominance of these bacteria in disinfectant and antiseptic solutions and the different strains exhibiting variable resistance to disinfectants have been reported earlier (Gambo *et al*., 2017). Other strains like *Proteus mirabilis* and *E. coli* have also been isolated and linked to nosocomial outbreaks amongst the major contaminants of their disinfectants (Keah *et al.,* 1995a). The high contamination rate highlights a lapse in infection control practices, with Gram-negative bacteria posing a greater threat due to their intrinsic resistance and biofilm-forming ability, which may compromise the disinfectant efficacy in clinical environments. These comparisons strengthen the evidence that contaminated disinfectants are a recurring issue in healthcare, reinforcing the need for routine quality control of biocidal agents in hospital settings

The fact that all the stock solutions of the disinfectants were not contaminated implied that the contamination probably arose during dilution or use. Various reports in scientific literature have linked the contamination of disinfectants in the hospital environment to suboptimal sanitary practices during the preparation and distribution of these biocides (Ogunsola *et al*., 2002). This suggests that contamination is not due to a manufacturer defect but rather in-hospital handling errors, indicating the need for better training of personnel and aseptic techniques during disinfectant preparation.

Also, it has been documented that diluted disinfectants prepared in hospital pharmacies for distribution to the various hospital departments and disinfectants that are in use in hospital departments have been exposed to contamination and that the effectiveness of disinfectants used in controlling nosocomial infection is often compromised by the fact that many of the disinfectants used in hospitals have been reported to be contaminated with organisms during preparation process (Weinstein, 2001).

The results of the antibiotic sensitivity tests presented in Table 2a indicate that the isolated bacterial strains exhibited varying degrees of susceptibility to the 17 antibiotics used in the study. Overall, 81% of the isolates demonstrated sensitivity to all tested antibiotics. However, 8 isolates exhibited resistance to three or fewer antibiotics, while 9 isolates were fully sensitive to all 17 antibiotics.

Table 1. Frequency of occurrence of the bacteria isolates.

Bacteria Identified	Frequency of occurrence	Percentage (%)
Pseudomonas aeruginosa	5	24
Klebsiella pneumoniae	4	19
E. coli	3	14.24
Proteus vulgaris	3	14.24
Salmonella typhi	3	14.24
Staphylococcus aureus	3	14.24
Total	21	100

Formula: Percentage (%) = (Total number of isolates/Frequency of occurrence) ×100.

Table 2a. Bacteria susceptibility testing result before curing.

Isolates/Antibiotics	CPX	OFX	LEV	CEP	PEF	CN	AU	E	AML	NB	S	APX	RD	CH	NA	PN	SXT	No of Rs	Sources of isolates
Pseudomonas	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0	HAM
Pseudomonas	S	S	S	S	S	S	S	S	R	R	S	R	R	R	R	R	R	8	HASW
Pseudomonas	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0	HDM
Klebsiella	S	S	S	S	S	S	R	S	R	R	S	R	R	R	R	R	R	9	HDSW
E. coli	S	S	S	S	S	S	S	S	S	R	S	R	R	R	R	R	R	7	HAM
E. coli	S	S	S	S	S	S	S	S	S	S	S	S	S	S	I	I	S	0	HAML
Proteus	S	S	S	S	R	S	R	S	S	S	R	S	S	S	S	S	S	3	HAML
Klebsiella	S	S	S	S	S	S	S	S	S	S	S	S	S	S	I	I	S	0	HAPW
Proteus	S	S	S	S	R	S	R	S	S	S	R	S	S	S	S	S	S	3	HASW
Pseudomonas	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	1	HASW
Klebsiella	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0	HBM
Proteus	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0	HBML
Salmonella typhi	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0	HBSW
Klebsiella	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0	HCM
Staph aureus	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	S	2	HCML
Staph aureus	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	S	2	HCSW
Salmonella typhi	S	R	S	R	R	R	R	I	R	R	R	R	R	R	R	R	R	15	HDM
Salmonella typhi	S	S	S	S	R	S	R	S	S	S	R	S	S	S	S	I	I	3	HDML
Staph aureus	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	S	2	HDPW
E. coli	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	R	S	2	HDSW
Pseudomonas	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	0	HDSW

Table 2b. Bacteria isolates selected for plasmid curing testing.

Isolates/Antibiotics	Source of Isolate	Total No of Antibiotics tested	No. of Antibiotic Resisted	Percentage (%) Resistant exhibited
Salmonella typhi	HDM	17	15	88
Klebsiella pneumoniae	HDSW	17	9	53
Pseudomonas aeruginosa	HASW	17	8	47
E. coli	HAM	17	7	41

Formula: Percentage Resistance= (Total Antibiotics Tested/No. of Antibiotics Resisted) ×100.

Table 3. Percentage sensitivity of test bacteria before and after plasmid curing.

Antibiotics /Isolates	Salmonella (Hosp D)		Klebsiella (Hosp D)		Pseudomonas (Hosp A)		E-coli (Hosp A)	
	Before Curing	After curing	Before Curing	After curing	Before Curing	After curing	Before Curing	After curing
OFX	R	R	NC	NC	NC	NC	NC	NC
CEP	R	R	NC	NC	NC	NC	NC	NC
PEF	R	R	NC	NC	NC	NC	NC	NC
CN	R	R	NC	NC	NC	NC	NC	NC
AU	R	R	R	R	NC	NC	NC	NC
E	R	S	NC	NC	NC	NC	NC	NC
AML	R	S	R	R	R	R	NC	NC
NB	R	S	R	S	R	R	R	S
S	R	S	NC	NC	NC	NC	NC	NC
APX	R	R	R	R	R	R	R	R
RD	R	R	R	R	R	R	R	R
CH	R	R	R	R	R	S	R	S
NA	R	R	R	R	R	R	R	R
PN	R	R	R	R	R	R	R	R
SXT	R	S	R	S	R	S	R	S
Total	15 R (100%)	10R & 5S (67%)	9R (100%)	7R & 2S (78%)	8R (100%)	7R & 1S (88%)	7R (100%)	4R & 3S (57%)

Notably, 19% (4 out of 21 isolates)—identified as *Pseudomonas aeruginosa, Salmonella typhi, Klebsiella pneumoniae,* and *Escherichia coli*—displayed resistance to more than six antibiotics. The identification of multidrug-resistant (MDR) strains in disinfectant-contaminated environments is alarming, as these may serve as reservoirs for hospital-acquired infections and limit therapeutic options.

The resistance patterns detailed in Table 2b reveal that among the 21 bacterial isolates, the following strains exhibited high-level antibiotic resistance: *Salmonella typhi* was resistant to 15 of 17 antibiotics, *Klebsiella pneumoniae* was resistant to 9 of 17 antibiotics, *Pseudomonas aeruginosa* was resistant to 8 of 17 antibiotics and *Escherichia coli* was resistant to 7 of 17 antibiotics.

Table 3 summarizes the resistance profiles before and after plasmid curing and isolates exhibiting resistance to more than six antibiotics (46% of the resistant strains): *Salmonella typhi* was

initially resistant to 15 antibiotics (100%), but sensitivity improved for 5 antibiotics, reducing resistance to 67%, *Klebsiella pneumoniae was i*nitially resistant to 9 antibiotics (100%), but sensitivity improved for 2 antibiotics, reducing resistance to 78%, *Pseudomonas aeruginosa was i*nitially resistant to 8 antibiotics (100%), but sensitivity improved for 1 antibiotic, reducing resistance to 88% and *Escherichia coli:* Initially resistant to 7 antibiotics (100%), but sensitivity improved for 3 antibiotics, reducing resistance to 57%. The findings indicate that antibiotic resistance in these isolates was mediated through plasmid-borne mechanisms. These findings align with observations that resistance to specific antibiotic, such as chloramphenicol can be plasmid-mediated, while resistance to others may persist due to chromosomal factors (Gambo *et al.,* 2024). These reductions indicate that plasmid-mediated resistance plays a significant role in the multidrug resistance of these strains, offering potential for future strategies that target plasmid stability or transmission to curb resistance.

*Pseudomonas aeruginosa r*esistance to Chloramphenicol and Septrin was lost after plasmid curing, suggesting plasmid-mediated resistance. However, resistance to Amoxil®, Norfloxacin, Ampiclox®, Rifampicin, Nalidixic Acid, and Ampicillin persisted, indicating resistance was not plasmid mediated. *Salmonella typhi r*esistance to 5 antibiotics was plasmid-mediated, whereas resistance to the remaining 10 antibiotics persisted, indicating resistance was not plasmid mediated. *Klebsiella pneumoniae s*ensitivity to Norfloxacin and Septrin improved post-curing, implying plasmid-borne resistance. Resistance to 7 antibiotics remained unchanged, indicating resistance was not plasmid mediated. *Escherichia coli* resistance to 3 was plasmid-mediated, whereas resistance to 4 antibiotics persisted tested post-curing, indicating resistance was not plasmid mediated. These findings align with observations that resistance to specific antibiotics, such as chloramphenicol and co-trimoxazole, can be plasmid-mediated, while resistance to others may persist due to chromosomal factors (Shuaibu *et al.,* 2016).

A study conducted in Kuwait found that 45% of *Salmonella Typhi* isolates exhibited multidrug resistance, particularly to ampicillin, chloramphenicol, tetracycline, and co-trimoxazole. The resistance traits were transferable to *Escherichia coli* recipients, indicating plasmid-mediated resistance (Panigrahi *et al*., 1996).

These findings underscore the growing challenge of antibiotic resistance, particularly in nosocomial infections. The study highlights that *Salmonella typhi*, a known pathogen in hospital-acquired infections, retained resistance to 15 out of 17 antibiotics, leaving only 2 effective treatment options. This raises concerns over the dwindling efficacy of antibiotics against infectious diseases, reinforcing the urgent need for antimicrobial stewardship programs (Ayliffe *et al.,* 1967). Additionally, *Pseudomonas aeruginosa* remains a significant opportunistic pathogen due to its intrinsic and acquired resistance mechanisms, including plasmid-mediated resistance factors. It is the most isolated Gram-negative bacterium in hospital-acquired infections, particularly affecting immunocompromised, neutropenic, and burn patients (Ogunsola *et al.,* 2002; Panigrahi *et al*., 1996).

A study conducted in six Malaysian hospitals found a high level of contamination in diluted disinfectants, suggesting that recommendations for cleaning disinfectant containers before refilling, handling diluted stock solutions, and using disinfectants were not closely adhered to. The study revealed that 91% of gram-negative with Pseudomonas species being the commonest, constituting 67.2% of all contaminant isolates. Gram-positive organisms made up the remaining 9.0% isolates. All the Pseudomonas spp. were resistant to gentamicin, ceftazidime, nalidixic acid and pefloxacin (Keah *et al.,* 1995b). Given its multidrug-resistant nature, infections caused by *Pseudomonas aeruginosa* pose a serious therapeutic challenge, emphasising the need for novel treatment strategies and continuous surveillance of antibiotic resistance patterns (Beier *et al*., 2015).

Conclusion

This study revealed that Gram-negative bacteria dominated disinfectant contamination in hospital settings, with *Pseudomonas aeruginosa* (24%) being the most prevalent, followed by *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus vulgaris*, *Salmonella typhi*, and *Staphylococcus aureus* (each 14%). Prior to plasmid curing, multidrug resistance was observed in *Salmonella typhi* (15 antibiotics), *Klebsiella pneumoniae* (9), *Pseudomonas aeruginosa* (8), and *E. coli* (7), all at 100% resistance levels. Post-curing resistance significantly declined for *Salmonella typhi* became sensitive to 5 antibiotics, *Klebsiella pneumoniae* to 2, *Pseudomonas aeruginosa* to 1, and *E. coli* to 3, indicating reductions of 67%, 78%, 88%, and 57%, respectively.

These findings confirm that resistance to specific antibiotics, particularly erythromycin, amoxicillin, norfloxacin, chloramphenicol, and septrin, was partially plasmid-mediated. Therefore, cross-resistance from disinfectant isolates to antibiotics is indeed linked to plasmid presence. This raises serious concerns, as plasmid-borne resistance genes can be horizontally transferred to other bacteria, facilitating the spread of antimicrobial resistance within clinical environments.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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