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EMERGENCE OF PLASMID-MEDIATED QUINOLONE RESISTANCE (PMQR) DETERMINANT FROM CLINICAL ISOLATES OF *PSEUDOMONAS AERUGINOSA*

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ABSTRACT

Introduction: Due to intrinsic resistance of Pseudomonas aeruginosa to many antibacterial agents, treatment of pseudomonal infections are difficult. Ciprofloxacin is the most effective quinolone against P. aeruginosa infection. However resistance developing to quinolones may become an important problem and is often a result of chromosomal mutations and by the effect of efflux pumps. Although there are reports on the plasmid-mediated quinolone resistance (PMQR) inthe members of Enterobacteriaceae family, they have not been detected in P. aeruginosa isolates until now. Hence this study was designed to determine the PMQR determinant in P. aeruginosa. **Objectives:** The aim of the study was to investigate the occurrence of PMQR determinant in clinical isolates of P. aeruginosa by phenotypic and molecular methods. **Materials and Methods:** A total of 34 strains of P. aeruginosa isolated from various clinical specimens such as pus (82.3%), urine (14.7%) and blood (2.9%) were included in the study. All isolates were tested for susceptibility to the following fluroquinolones: ciprofloxacin, levofloxacin and ofloxacin by Kirby Bauer disc diffusion method. MIC of ciprofloxacin was determined by agar dilution method according to CLSI guidelines. DNA extraction was done by boiling lysis method. Molecular detection of PMQR determinants (qnr A, qnr B and qnr S) was done for all the isolates. **Results:** Disc diffusion tests showed resistance to ciprofloxacin, levofloxacin and ofloxacin. Of the 34 isolates screened, qnr A and qnr S genes were not detected in any of the tested isolates. 25(73.5%) isolates were positive for qnr B gene and these isolates showed MIC ranging from 32 µg/ml to 256µg/ml. **Conclusion:** Plasmid mediated quinolone resistance genes have so far not been identified in P. aeruginosa. However, recent studies have detected qnr B in P. fluorescens and P. putida. This is the first report of qnr B gene identified in P. aeruginosa clinical isolates.

KEYWORDS: P. aeruginosa, fluroquinolones: ciprofloxacin, levofloxacin and ofloxacin.

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacterium frequently found in soil, marine habitats, plants, animals and humans. P. aeruginosa is frequently present in small numbers in the normal intestinal flora and on the skin of humans and is the major pathogen of the group. Other Pseudomonads infrequently cause disease. (Stover et al., 2000). The ability of P. aeruginosa to survive on minimal nutritional requirements and to tolerate a variety of physical conditions has allowed this organism to persist in both community and hospital settings. P. aeruginosa is a member of the normal microbial flora in humans. Representative colonization rates for specific sites in humans are 0 to 2% for skin, 0 to 3.3% for the nasal mucosa, 0 to 6.6% for the throat and 2.6 to 24% for fecal samples. However, colonization rates may exceed 50% during hospitalization, especially among patients who have experienced trauma to or a breach in cutaneous or mucosal barriers by mechanical ventilation, tracheostomy, catheters, surgery or severe burns(Lister et al., 2009).

A data collected by the CDC National Nosocomial Infections Surveillance System from 1986 to 1998 showed that P. aeruginosa was identified as the fifth most frequently isolated nosocomial pathogen, accounting for 9% of all hospital-acquired infections in the United States (Emori et al., 1993). P. aeruginosa was also the second leading cause of nosocomial pneumonia (14 to 16%), third most common cause of urinary tract infections (7 to 11%), fourth most frequently isolated pathogen in surgical site infections (8%) and seventh

leading contributor to bloodstream infections (2 to 6%). Data from more recent studies continue to show P.aeruginosa as the second most common cause of nosocomial pneumonia, health care- associated pneumonia, and ventilator-associated pneumonia (Weinstein et al., 2005) and the leading cause of pneumonia among paediatric patients in the intensive care unit (ICU)(Lister et al., 2009).

The *Pseudomonads* are gram-negative bacilli, motile, obligate aerobe measuring about 0.6 x 2µm some produce water-soluble pigments.*P. aeruginosa* grows on many types of culture media, sometimes producing a sweet or grapelike or corn taco like odour. They are beta haemolytic and forms smooth round colonies with a fluorescent greenish colour. It often produces the nonfluorescent bluish pigment pyocyanin, which diffuses into the agar. Many strains of *P. aeruginosa* also produce the fluorescent pigment pyoverdin, which gives a greenish colour to the agar. Some strains produce the dark red pigment pyorubin or the black pigment pyomelanin. . Cultures from patients with cystic fibrosis (CF) often yield *P. aeruginosa* organisms that form mucoid colonies as a result of overproduction of alginate, an exopolysaccharide. In CF patients, the exopolysaccharide appears to provide the matrix for the organisms to live in a biofilm(Brooks et al., 1998).
Kingdom: Bacteria
Phylum: Proteobacteria
Class: Gammaproteobacteria
Order: Pseudomonadales
Family: Pseudomonadaceae
Genus: *Pseudomonas*
Species: *aeruginosa*

Each *P. aeruginosa* cell possesses a single polar flagellum and several much shorter type 4 pili localized at a cell pole. These are proteinaceous appendages function both as adhesins and as major means of motility. Flagella and pili can also initiate an inflammatory response. Type 4 pili are the most important adhesins of *P. aeruginosa* and are also involved in twitching motility and the formation of biofilms. They extend and retract to pull the cell along solid surfaces by a process termed 'twitching motility' (Kipnis *et al.,* 2006). Together with flagella, pili also facilitate swarming motility, a highly coordinated form of motility on semisolid surfaces (Kohler *et al.*,2000; Yeung *et al.,* 2009).

Type 3 secretion system (T3SS) are shared among many pathogenic Gram negative bacteria as a means of injecting toxins directly into host cells. The *P. aeruginosa* T3SS is a major determinant of virulence, and its expression is frequently associated with acute invasive infections and hasbeen linked to increased mortality in infected patients (Sadikot *et al.,* 2005; Hauser, 2009).

Quorum sensing (QS) is a mechanism shared by many bacteria that allows for a coordinated adaptation of a bacterial population to environmental changes, including the adaptation to the lung environment. This adaptation is mediated by small membrane diffusible molecules called autoinducers. which are cofactors of specific transcriptional regulators. The concentration of autoinducer molecules in the medium is proportional to the concentration of bacteria and when the bacterial population increases to a critical mass (i.e. 'quorum'), and the concentration of autoinducers becomes sufficient to cause activation of specified downstream genes resulting in a coordinated response across the entire bacterial population. It is estimated that as many as 10% of genes in the genome and more than 20% of the expressed bacterial proteome are regulated by QS (Deep *et al.,* 2011). Biofilms are highly organized, structured communities of bacteria attached to one another and to a surface, and their formation is intricately linked to QS (Bjarnsholt *et al.,* 2010). These communities are encased in extracellular polymeric substances (EPS) that can consist of polysaccharides, nucleic acids, lipids, and proteins. The EPS matrix makes up the majority (50–90%) of the volume of the biofilm and imparts both a physical and chemical robustness to the community by resisting mechanical forces (e.g. flowing water) and decreasing the penetration of toxic chemicals (e.g. antibiotics, host defence molecules); Hall-Stoodley & Stoodley, 2009; Lieleg *et al.,* 2011).

The transition of *P. aeruginosa* from the motile to sessile state in biofilms, and back again, manifests itself as a multitude of physiological changes. Several proteases are secreted by *P. aeruginosa*. These proteases have established roles in ocular infections and in sepsis, where they can degrade immunoglobulins and fibrin and disrupt epithelial tight junctions (Kipnis *et al.,* 2006). While their contribution to lung infections is less clear, proteases have been shown to contribute to tissue damage in respiratory infections, including the degradation of host lung surfactant (Fleiszig & Evans, 2002; Hobden, 2002;Kipnis *et al.,* 2006). Alkaline protease is a type 1 secreted zinc metalloprotease that is known for its degradation of host complement proteins and host fibronectin (Laarman *et al.,* 2012).*P. aeruginosa* produces two elastases LasA and LasB, which are regulated by the las I quorum sensing system and secreted via type 2 secretion systems (Toder *et al.,* 1994; de Kievit & Iglewski 2000). Most *P. aeruginosa* investigations reserve the term 'elastase' for LasB and 'staphylolysin' for LasA. This is because LasA, a serine protease, is able to hydrolyze the penta glycine bridge required for peptidoglycan stabilization in the cell wall of staphylococci, but has only a fraction of the elastolytic abilities of LasB and rather is thought to enhance the proteolytic activity of LasB (Toder *et al.,* 1994; Matsumoto, 2004).

Protease IV is a serine protease that can degrade complement proteins, immunoglobulins, and fibrinogen.

Injections of protease IV onto the cornea in a rabbit model of ocular infection caused erosion of the corneal epithelium, while infection of corneas with a protease IV deficient strain showed reduced virulence (Engel *et al.*, 1998).

Lipopolysaccharide is a complex glycolipid that forms the outer leaflet of the outer membrane and has roles in antigenicity, the inflammatory response, exclusion of external molecules, and in mediating interactions with antibiotics (King *et al.*, 2009). *P. aeruginosa* produces a three domain lipopolysaccharide consisting of a membrane anchored lipid A, polysaccharide core region, and a highly variable O-specific polysaccharide (O-antigen or O-polysaccharide). The importance of lipopolysaccharide to the bacterium and to host pathology and antibiotic resistance has subjected it to intense study and a great deal is now known about its biosynthesis and the contributions of its structural domains to the above observations.

Lipid A is an atypical glycolipid that anchors the lipopolysaccharide into the outer membraneand is composed of a diglucosamine biphosphate backbone with O and N linked primary and secondary fatty acids. (Lam *et al.*, 2011).

In wild-type strains, the lipid A domain is attached to a conserved nine or ten sugar, branched oligosaccharide core. ('O-antigen'). Two types of O-antigen can exist simultaneously within a given cell, and they are distinct structurally and serologically. A band ('common') polysaccharide is a homopolymer of D-rhamnose approximately 70 sugars long and which elicits a weak antibody response. In contrast, B-band ('O-specific') polysaccharide is a strain variable heteropolymer both in chain length and in the nature of the sugars, and this lipopolysaccharide elicits a strong antibody response and is the chemical basis for serotyping (King *et al.*, 2009).

Infections by *P. aeruginosa* are difficult to treat due to its intrinsic ability to resist many classes of antibiotics as well as its ability to acquire resistance. All known mechanisms of antibiotic resistance can be displayed by this bacterium (intrinsic, acquired, and adaptive); sometimes all within the same isolate. Resistance rates are on the rise despite the use of combination drug therapies (Moore & Flaws, 2011). As few new drugs are available to combat *P. aeruginosa* infections, there has been a return to the use of older drugs such as polymyxins (Livermore, 2002) Intrinsic resistance is encoded in the microorganism's chromosome. In the case of P. aeruginosa, intrinsic resistance is due to the low permeability of its outer membrane, the constitutive expression of membrane efflux (Mex) pumps, and the natural occurrence of an inducible chromosomalβ-lactamase, AmpC (Strateva & Yordanov, 2009). The outer membrane is a semi permeable barrier that restricts the uptake of small hydrophilic molecules such as β-lactam antibiotics *P. aeruginosa* outer membrane is 10 to 100fold less permeable than that of *E. coli*, having fewer large channel porins (formed by OprF) and a number of small channel porins (formed by proteins such as OprD and OprB; Breidenstein *et al.*, 2011).

These efflux pumps can eject a wide range of antibiotics; for example, MexAB–OprM and MexXY–OprM can collectively efflux β-lactams, chloramphenicol, fluoroquinolones, macrolides, novobiocin, sulfonamides, tetracycline, and trimethoprim and aminoglycosides (Livermore, 2002; Schweizer, 2003). Mutations that result in alterations of an antibiotic's target can also confer resistance, where a mutation in DNA gyrase reduces the binding affinity of the enzyme for fluoroquinolones leading to resistance (Schweizer, 2003;Breidenstein *et al.*, 2011).

qnr GENE

Quinolones, especially ciprofloxacin, are important for the treatment of P.aeruginosa infections, but resistance to quinolones is known to occur and is often a result of chromosomal mutations and by the effect of efflux pumps. Recently plasmid-mediated quinolone resistance have been reportedin the members of Enterobacteriaceae family. The gene responsible for this resistance is called qnr.

The plasmid-borne quinolone resistance gene *qnr* was reported in 1998 and comprise three families, *qnrA*, *qnrB*, and *qnrS*, differing from each other 40% or more in nucleotide sequence. Within each family, minor (≤10%) variation in sequence has defined a growing number of alleles.

After identification of the qnr genes, different sub-types (*qnrA*, *qnrB*, *qnrC*, *qnrS* and qnrD) have also been definedThe first *qnr* gene, now known as *qnrA*, was found to protect *E. coli* DNA gyrase from inhibition by ciprofloxacin. Subsequent research demonstrated that *qnr* genes co-occur with other resistance determinants and *qnr* determinants have been reported in bacterial strains producing extended spectrum β-lactamases.

Thedistribution of *qnr* genes suggests that they have been in existence for some time. The *qnrA* gene is thought to have originated in*Shewanella* algae, an environmental species from marine and fresh water. Qnr-like proteins have been detected in water-borne *Vibrionaceae* bacterial isolates and may be the origin of the more clinically relevant *qnrA*, *qnrS*, and *qnrB* determinants, with which they share 40–67% homology.

A recently proposed consensus for *qnr* nomenclature defined *qnr* as a naturally occurring allele encoding a pentapeptide repeat protein that confers reduced susceptibility to nalidixic acid or a fluoroquinolone. *qnr*families (such as *qnrA*, *qnrB*,

or *qnrC*) are defined by a 30% or more difference in nucleotides or derived amino acids.

Thus, the present study focusses on the resistance pattern of P. aeruginosa isolates againstciprofloxacin, levofloxacin, ofloxacin and also the molecular detection of qnrA, qnrB and qnrS genes in these isolates**.**

The main aim of this study: 1. To perform antibiotic susceptibility testing (AST) for the P. aeruginosa isolates against ciprofloxacin, novofloxacin and ofloxacin. 2. To perform minimum inhibitory concentration testing for the ciprofloxacin resistant isolates by agar dilution method. 3. To detect the presence of qnrA, qnrB, qnrS genes in the P. aeruginosa isolates by multiplex polymerase chain reaction.

MATERIALS AND METHODS

SOURCE OF ISOLATES

A total of 34 strains of *Pseudomonas aeruginosa* isolated from various clinical specimens were included in the study (Figure 1).

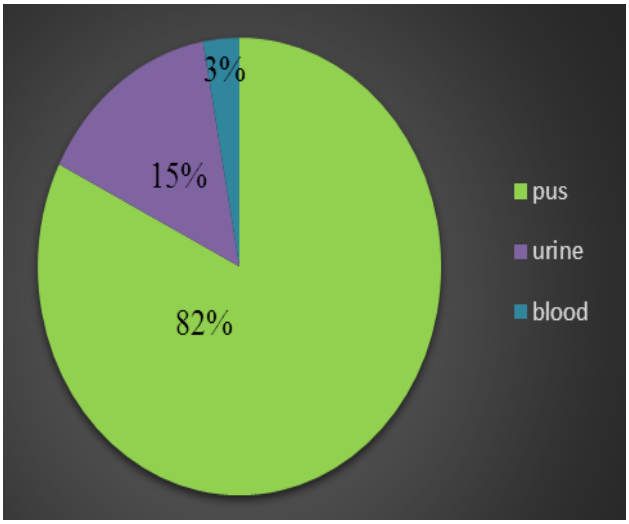

Figure 1: Sources of *P.aeruginosa* isolates.

ANTIMICROBIAL SUSCEPTIBILITY TESTING (AST)

All the strains were tested against ciprofloxacin, levofloxacin and ofloxacin by disc diffusion method and interpreted according to CLSI guidelines 2013.

MINIMUM INHIBITARY CONCENTRATION (MIC)

Table 1: Preparation of antibiotic stock solutions.

Dilutions	Stock solutions	Volume of the stock to be added to 100 ml of agar	Final concentration of antibiotic in agar (µg/ml)
1	**Stock A**: 10,000µg/ml	2.56	256
2		1.8	128
3		0.64	64
4		0.32	32
5		0.16	16
6	**Stock B**: 1,000µg/ml	0.8	8
7		0.4	4
8		0.2	2
9		0.1	1
10	**Stock C**: 100µg/ml	0.5	0.5

MIC of ciprofloxacin was determined by agar dilution method with concentration ranging from 0.5 µg/ml to 256 µg/ml according to CLSI guidelines 2013(Table 1).

DNA EXTRACTION

DNA extraction was performed by boiling lysis method. Overnight culture of *P. aeruginosa* isolates in LB broth were centrifuged at 10,000 rpm for 10 minute. To the pellet, 300 µl of PCR water was added and boiled at 100°C for 10 minutes, immediately kept at -20^{o} C for a minimum of 6hrs and centrifuged at 10,000 rpm for 10 minute. After centrifugation supernatant was stored at -20°C.

POLYMERASE CHAIN REACTION

Multiplex PCR was performed for all the 32 isolates using the following primers.

Gene	Primer (5' ⟶ 3')	Tm	Amplicon Size
qnrA	F-TTCAGCAAGAGGATTTCTCA R-GGCAGCACTATTACTCCCAA	55	670bp
qnrB	F-CCTGAGCGGCACTGAATTTAT R-GTTTGCTGCTCGCCAGTCGA	60	402bp
qnrS	F-CAATCATACATATCGGCACC R-TCAGGATAAACAACAATACCC	55	602 bp

PCR REACTION MIX (25 µl)

PCR reagents	Volume (µl)
10X PCR buffer	2.5
dNTP mixture(2.5mm conc.)	0.5
Forward primer(10picomolar)	0.5
Reverse primer(10picomolar)	0.5
Taq polymerase(0.75 unit)	0.25
PCR water	18.75
Template DNA	2

PCR CONDITIONS

Initial Denaturation-94 for 5 min
Denaturation -94 for 30 secs
Annealing temp-60 for 45 sec
Extension -72 for 50 secs
Final extension-72 for 5 min
} 30cycles

DETECTION OF PCR PRODUCT

The resulted PCR products were resolved on 2% agarose gel stained with Ethidium bromide, (4µg/ml) using a 100bp DNA ladder as a size marker and visualized in UV transilluminator carestream 212 PRO.

RESULTS

Disc diffusion results

29/35 isolates were resistant and 6 isolates showed intermediate resistance to ciprofloxacin; 30/35 isolates were resistant, 4 isolates showed intermediate resistance and 1 isolate was susceptible to levofloxacin; 34/35 isolates were resistant and 1 isolate was intermediately resistant to ofloxacin (Figure2).

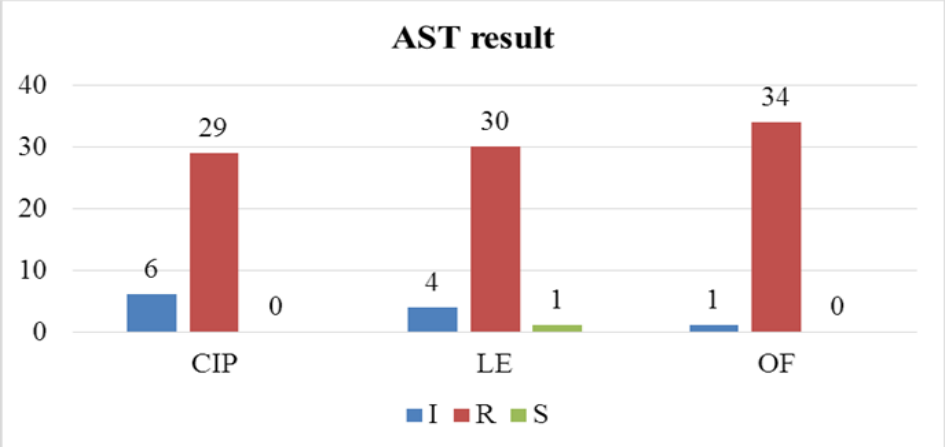

Figure 2: AST result of Ciprofloxacin, Levofloxacin and Ofloxacin.

MIC results

With regards to ciprofloxacin, resistance was observed in 16 isolates, intermediate resistance in 4 isolates and susceptibility in 10 isolates.

PCR results

The *qnrA* and *qnrS* were not detected in any of the tested *P. aeruginosa* isolates. The *qnrB* gene was detected in 24 (70.5%) isolates among the 34 isolates.

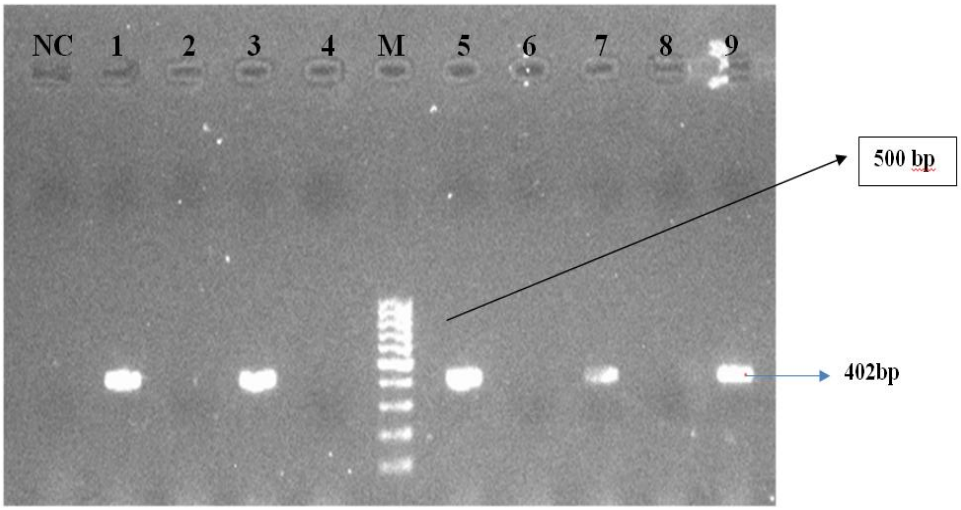

Figure 3: Representative gel pictures of *qnrB* gene.
Lane 1- Negative control; Lane M-100bp ladder; Lane2-9 clinical isolates

DISCUSSION

P. aeruginosa is an important infectious agent that is usually associated with health care settings causes a variety of clinical manifestations in hospital patients, especially among immunocompromised individuals (Gayyurhan E *et al*., 2008*). P. aeruginosa* is responsible for 10% to 25% of hospital acquired infections with high mortality and morbidity rate (Gul M E *et al*., 2004). Beta–lactam antibiotics with anti-pseudomonal activity are often administered for treatment. Quinolones especially ciprofloxacin, also have antipseudomonal activity that demonstrate bactericidal effect. Quinolone use is a significant risk factor for selecting of resistant *P. aeruginosa* in individual patients and in population.

Plasmid mediated quinolone resistance encoded by the qnr gene have different sub type (*qnrA, qnrB, qnrC, qnrS* and *qnrD*). The main mechanisms for quinolone resistance are chromosomal mutations in DNA gyrase and topoisomerase IV enzymes, and decrease drug accumulation within the cell through hyper activation of efflux pumps and reduced cell wall permeability (Ruiz J. *et al.*, 2003).

However, numerous additional *qnr* genes have recently been identified, suggesting that they have existed in nature for many years. In a study that analyzed the sequence of 48 Gram-negative bacteria, *qnrA* (*qnrA3-qnrA5*) variants were detected in Shewanella algae chromosome. It was reported that the MIC value of this isolate was 4-8 fold greater than that of *S. putrefaciens*, which did not harbour this gene. *Shewanella spp*. can be found in marine and fresh water sources. The *qnrB5* and *qnrB19* genes were also identified in microbial population samples obtained from sea water. The *qnrS2* gene was also detected in *Aeromonas punctata* subsp. punctata and *A. media* isolates obtained from the Seine River.

The finding that fluoroquinolone resistance is most closely linked to levofloxacin use (and not to ciprofloxacin use) is intriguing. Rates of fluoroquinolone resistance in *P. aeruginosa* were increasing before levofloxacin was widely used, so use of this agent cannot be solely responsible. However, levofloxacin may be more likely to select for resistant strains of *P. aeruginosa* than is ciprofloxacin.

CONCLUSION

In this study, *qnrA* and *qnrS* genes were not detected in any of the *P. aeruginosa* isolates.The *qnrB* gene was detected in 24 of the 34 isolates.

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COMPETING INTERESTS

No competing interest declared.

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