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# Study the Effect of Biofilm Production on Antibiotic Resistance in Proteus mirabilis Isolated from Clinical Samples in Kirkuk City

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**A B S T R A C T**

*Proteus mirabilis* is one of the most infectious organisms that inhabits the environment and causes a various infections involving those of the skin, respiratory tract, wounds and urinary tract. One of the main virulence features in *P. mirabilis* that plays a role in the pathogenesis and antibiotic resistance is the ability to form biofilm. The aim of study was to determine the relation between the antibiotic resistance and the biofilm formation by *P. mirabilis*. A total of (205) samples were collected from different clinical samples (urine, wound and burn swabs) in Kirkuk city hospitals. (32) Isolates were identified as *P. mirabilis*. Eight antibiotic discs were used for the antibiotic sensitivity test by disc diffusion method. Also, a micro titter plate method used for the detection of biofilm. The results of the antibiotic sensitivity showed variation in the rates of antibiotic resistance, doxycycline 29(90.6%), Trimethoprim-sulfamethaxole 27(84.37%), Ampicillin 22(68.75%) and amoxicillin/clavulanic 21(65.62%) resistance were found in the majority of the isolates. Gentamicin and cefotaxim (37.5%, 40.6%) respectively were moderate resistance, while Imipenem and Ciprofloxacin have low resistance and consider the most effective medication against *P. mirabilis*. The results showed 8 (25%) of *P. mirabilis* strong biofilm producer, 13 (41.6%) moderate biofilm producer, 8 (25%) weak biofilm producer and 3 (9.37%) non-biofilm producer. The study concluded that there is a link among biofilm production and antibiotic resistance.

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## Introduction

Proteus mirabilis is a Gram-negative bacterium that belonging to the Enterobacteriaceae family and have the ability of changing in it is shape and using the flagella for swarming motility. It is causes several infections such as urinary tract infection, a middle ear infection, wounds and burn in addition to other infections[1,6]. It is an important opportunity bacterium that is found in water, soil and in the intestinal tracts of humans and animals. It has been recognized as a leading cause of urinary tract infections [2].

Proteus spp. has numerous virulence factors which aid in adhesion, growth, colonization and invasion into infected tissues and progressing of a pathogenesis. These virulence factors include flagella, capsule, fimbriae, outer membrane proteins, lipopolysaccharides (LPS), biofilm production, and several enzymes like, haemolysin, metalloprotease, amino acid deaminase, urease [3] and chondroitinase [3,4].

The global health problem is the antibiotic resistance that limits the treatment options. There are numerous mechanisms which bacteria can resist the antibiotics including; inactivation of antibiotic by bacterial enzyme, decrease antibiotic entry into the bacteria, antibiotic efflux as well as target site mutation [5]. Moreover, bacteria can develop multidrug resistance (MDR) this could lead to ineffective antibiotic therapy and helps in prevalence of persistent infections [1].

Biofilms are accumulation of cells that adhere to surface and are surrounded by a matrix of polysaccharides, proteins and the nucleic acids [2]. Bacteria within biofilm exhibit high levels of resistance to biocides and antibacterial agents. These agents are needed at high levels, which can be 1000- fold greater than that required in case of the planktonic cells to produce antibacterial effects [7]. The biofilms that form in the urinary tract, particularly in catheterized individuals, are the most widely investigated P. mirabilis biofilms [8].

Biofilm production in urinary tract infections causes long-term infection so, it supports both body defence and antibiotic resistance so, the accumulation of bacteria occur in epithelial cells and breakdown of these cells and produce of urease that transform urea to the ammonia lead to the formation of stone that prevent urine from passing through the urinary tract and cause polynephritis [33, 34].

In general, biofilm production increase bacterial resistance, increase the treatment period and aggravates of infection. As well as, protect the organisms from host immune system and the antimicrobial agents [8].

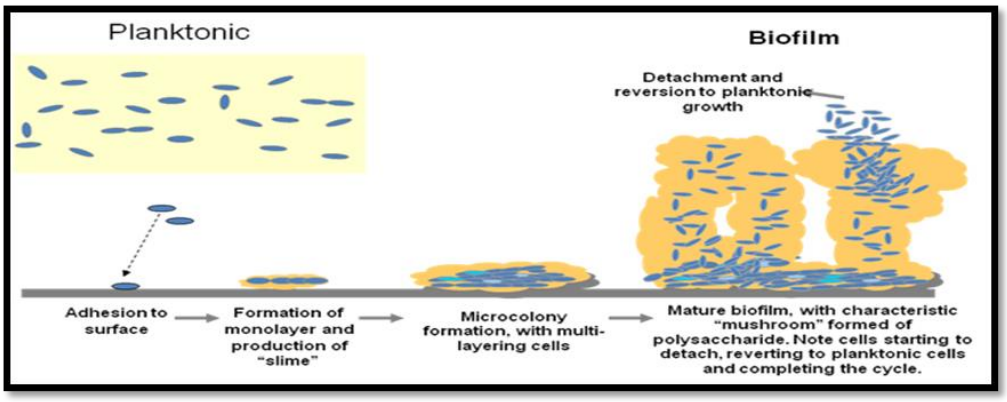


Figure 1. Various stages of biofilm production and development (Vasudevan 2014) [36]

## Materials and Methods

### 1. Ethical issue

Certain official agreement was taken from Kirkuk health directorate before establishing the study.

### 2. Study design an period

A descriptive correctional study was done on four hospitals named as: Kirkuk General Hospital, Azadi Teaching Hospital and General Paediatric Hospital which is from the period December 2021 to February 2022.

### 3. Study subject

A total of two hundred and five (205) samples thirty two (32) samples were identified as P. mirabilis.

## 4. Sampling collection

Total of two hundred and five (205) samples thirty two (32) samples were identified as P. mirabilis were collected from period December 2021 to February 2022 from different clinical samples urine, wound and burn swabs from patients attending to (Azadi teaching hospital, General Kirkuk hospital and Podiatric teaching hospital) in Kirkuk city.

## 5. Identification of the Isolates

Isolation of P. mirabilis was done by streaking of all samples on blood and MacConkey agar then incubated at 37°C for 24 hours. Bacterial identification was done by using biochemical test [8], and confirmed by the API 20E system.

## 6. Antibiotic Susceptibility Test

Eight antibiotic discs were used to detect the sensitivity of 32 isolates of P. mirabilis according to Bauer et al., (1996) method. Bacterial isolates were grown overnight at 37◦C on nutrient broth. Then prepared Muller Hinton agar and divided into Petri dishes. Isolated colonies were vortexed in 5ml of normal saline suspension. The turbidity of suspension was contrasted with McFarland standard. An aliquot of 2µL of bacterial suspension was placed on the Muller Hinton agar, spread with cotton swab in three different directions by rotating the plate 60◦ for each direction. The plate was turned upside down at room temperature for a few minutes. A variety of antibiotics were added on the plates then incubated at 37°C for 24 hours. A number of antibiotics were placed on the plates and incubated at 37◦C overnight. Zone of inhibition was measured in (mm) by zone inhibition ruler. The results were interpreted in according to National Committee for Clinical Laboratory Standards [12].

Table 1. Antibiotic discs used in this study

| No. | Antibiotics | Abbreviation | Concentration (µg) | Manufacture company/ origion |
|---|---|---|---|---|
| 1. | Amoxicillin / clavulanic | AMC | 30 | Bioanalyse/ Turkish |
| 2. | Ciprofloxacin | CIP | 5 | |
| 3. | Gentamicin | CN | 10 | |
| 4. | Doxycycline | DOX | 30 | |
| 5. | Ampicillin | AMP | 10 | |
| 6. | Trimethoprim-Sulfamethaxole | STX | 25 | |
| 7. | Imipenem | IMP | 10 | |
| 8. | Cefotaxim | CTX | 30 | |

## 7. Identification of biofilm by micro titter plate assay method

Thirty two isolates were put in to tryptone soy broth (TSB) media that had been added with 1% glucose (TSB glu) incubated for 24 hours at 37°C and then diluted 1 :100 with new medium. Each a micro titter plates well (out of 96 wells) was filled with 200 µL of the diluted culture and only broth was used as a control, incubation of Culture for 48 hours at 37°C. After incubation, the plate was gently washed with phosphate buffer (PBS) saline three times to eliminate unattached cells. Then 200µL 0.1% of crystal violet was added to each well and incubated for 10-15 minutes at room temperature, after that, crystal violet were removed from the plate by washing it three time with distilled water, micro titter plate turned upside down for few hours for drying. For resolubilizing the dye 200 µL of 95% ethanol solution was added to each wells and microtiter plate was covered with lid and kept at room temperature for 30 minutes. To measure the absorbance Enzyme Linked Immunosorbent reader used, optical density of every well measured at 630nm. To obtain optical results repeat this procedure three time [36]. Biofilm is interpreted as

ODi $\leq$ ODc = Non biofilm producer
ODc < OD $\leq$ 2 ODc = Weak biofilm producer
2 ODc < OD $\leq$ 4 ODc = Moderate biofilm producer
4 ODc < OD = Strong biofilm producer

## Results and Discussion

## *P. mirabilis* isolation and identification:

Two hundred and five (205) samples were collected from patient attending to (Azadi teaching hospital, General Kirkuk hospital and Podiatric teaching hospital) in Kirkuk city. These samples were distributed as follow: urine sample (133), wound swab (44) and burn swab (28) as shown in (table 1). Thirty two isolates were characterized depending on cultural and microscopic characteristic. Biochemical tests were used to genus and species characterization and API 20E used as a confirmatory test.

The isolates were first identified as belong to genus *Proteus* by the swarming phenomenon on n blood agar (figure 2), culture characteristic smell, and pale appearance of the bacteria (non-lactose fermented) on the MacConkey agar, also, examination of the bacteria by microscope, which appeared as straight rods and Gram negative when it stained with the Gram stain [15].

Many conventional biochemical tests were performed to characterize suspected Proteus isolates. All the thirty two *P. mirabilis* isolates showed positive results to the catalase, urease (figure 3), and motility, but were negative to indole citrate and oxidase test [16]**.** API 20E strip used for confirmation of biochemical results (figure 4) [5].

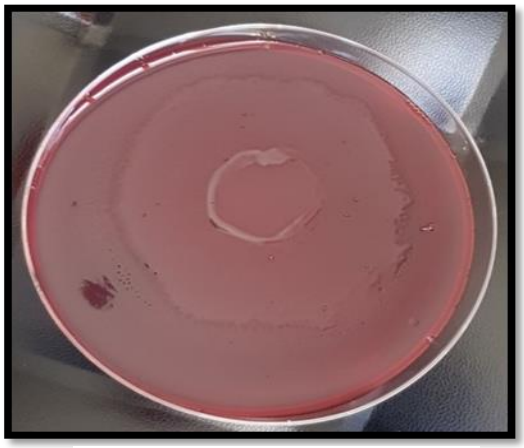

**Figure 2.** Swarming phenomenon on blood agar

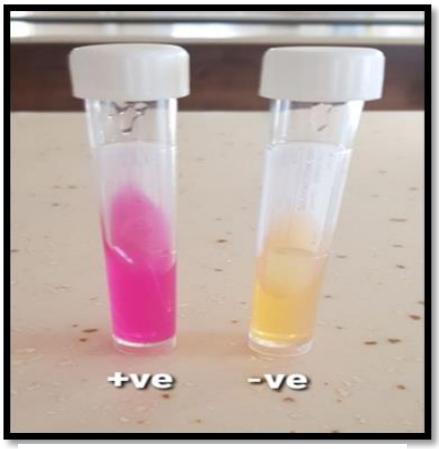


**Figure 3.** Urease test

**Figure 4.** API 20 E

**Table 2.** Isolation source and percentage of *Proteus mirabilis*

| Isolation Source | Number of samples | Number of *p. mirabilis* isolates | % of total samples |
|---|---|---|---|
| Urine | 133 | 23 | 11.2% |
| Wound | 44 | 6 | 2.9% |
| Burn | 28 | 3 | 1.5% |
| Total | 205 | 32 | 15.6% |

Two hundred and five clinical samples (urine, wound and burn swabs) were collected. It was found that 32 isolates (15.6%) were identified as P. mirabilis. The current result was agree with [17] who mentioned that P. mirabilis from clinical samples represented (12.6%), whereas the results were lesser than [18, 19] who mentioned that P. mirabilis from clinical samples represented (28%, 28.4%). Also the results of the current study showed that the higher percentage of P. mirabilis isolates was from urine samples represented as (11.2%) from total sample. The results were agree with [20], who mentioned that the highest source of isolation of P. mirabilis was isolated from the urine sample, In contrast with studies performed by [18] who mentioned that P. mirabilis isolated from wound swab more commonly than in other clinical samples. The explanation for the

variation in the percentages of isolation might be because of the differences in samples size, isolations source, and number of the hospitals that surveyed and the differences in collection season for samples.

## Antimicrobial susceptibility testing

The pattern of antibiotic resistance of the P. mirabilis isolates are shown in table 2. These isolates showed different levels of resistance to each antibiotic that were tested. Doxycycline has the highest rate of resistance 29(90.6%), followed by Trimethoprim-sulfamethaxole 27(84.37%), ampicillin 22(68.75%), amoxicillin/clavulanic 21(65.62%), cefotaxim 13(40.6%), and gentamycin 12(38%). While the most effective antibiotics against P. mirabilis imipenem 32 (100%) and ciprofloxacin 22 (68.75%).

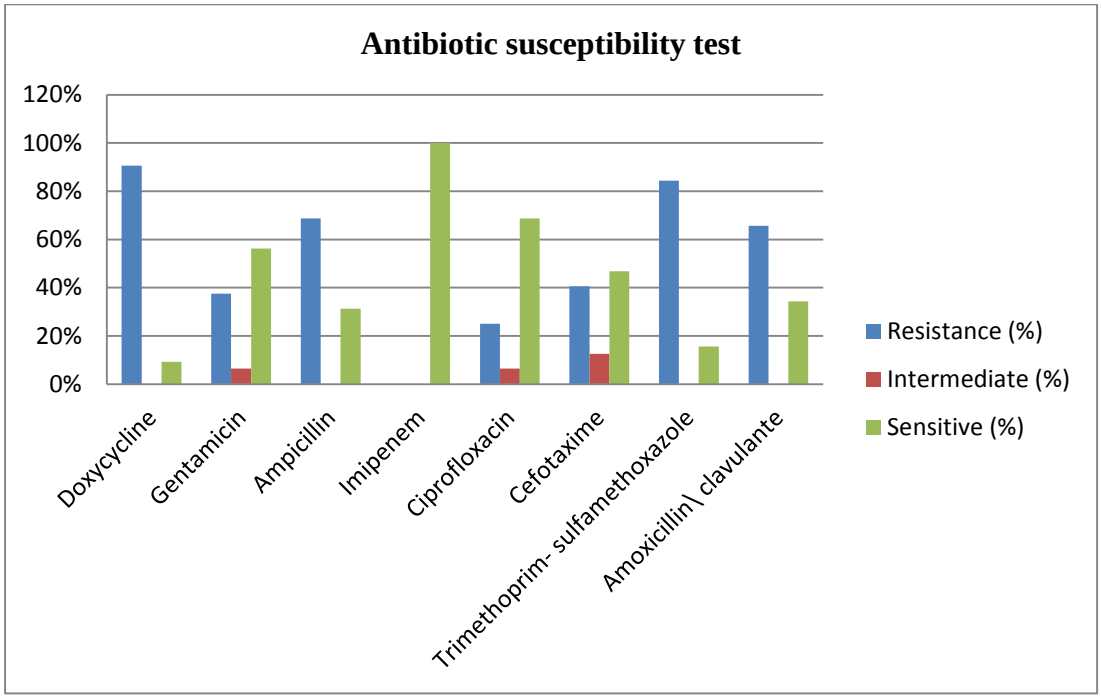


**Figure 5.** Antibiotic susceptibility test pattern *P. mirabilis*

Results shown in (table 2) the (32) isolates of P. mirabilis have the highest antibiotic resistance toward Doxycycline as it reached (90.6%). The resistance rate was agree with the study results with [31] recorded (95%). In contrast another study recorded lower resistance 43.1% [19].

Gentamycin is an aminoglycoside antibiotic which is broad-spectrum and inhibitor for protein synthesis. In this study, P. mirabilis isolates show moderate resistance to gentamycin (37.5%). This result agree with the researcher [19] recorded 41.2% while disagrees with [31] were the isolates show low resistance (7.5%).

Bacterial resistance to Ampicillin in this study is (68.75%), which agree with [31] who recorded the resistance to P. mirabilis as (67.5%). In contrast another study recorded high resistance 85.1% [2].

As for the carbapenems represented by Imipenem, the percentage of resistance of the isolates against this antibiotic in the current study was (0%). This result was close to the study conducted by [21] who also recorded the resistance to imipenem as (0%). The results of the current study differ with what was reached by [25] as the percentage of resistance was (25%). Imipenem is the most effective antibiotic used in this study its sensitivity because of its stability not affected by beta-lactamase enzyme and high rate of permeability through the bacterial outer membrane.

Ciprofloxacin belongs to the fluroquinolone medication class and is a synthetic chemotherapeutic antibiotic [26]. Ciprofloxacin resistance was found in (25%) of the isolates in this study. These results agree with [24], who found that the resistance of the isolates were (38.3%). In contrast to result reported by [27] with lower resistance to Ciprofloxacin which is (0%). While [21, 28] recorded high resistance of isolates toward this antibiotic (70% and 53.3%) respectively. The high resistance of P. mirabilis isolates toward Ciprofloxacin may be due to a genetic mutation that leads to a change in the target site and thus will prevent the binding of the antibiotic to it, or it may be due to an increase in the flow systems [29] and efflux pump systems.

Third generation cephalosporin represented by Cefotaxim the isolates against this antibiotic in the current study was (40.6%). This result was agree to the study reported by [30, 2] and found that (35% and 51.1%) respectively. While disagree with what was reached by [17] as the percentage of resistance was higher than the current study (65%).

Antibiotic resistance toward trimethoprim-Sulphamethazole in the current study was (84.37%) The resistance rate was agree with the study results with [28, 2] who found that the resistance of the isolates were (82%, 78%) respectively, While [20] recorded lower resistance of isolates toward this antibiotic (71.8%).

Amoxicillin/clavulanic resistance recorded as (65.62%). This result agrees with [31] who recorded as (65%) respectively, while disagree with the studies conducted by [27, 28] who recorded as (83.7%, 90%) respectively.

## Biofilm production

A total of (32) P. mirabilis isolates were tested, 29 (90.6%) were biofilm producer while 3 (9.37%) were non biofilm producer. For the biofilm producing 13 (40.6%) were moderate biofilm producers, whereas 8 (25%) and 8 (25%) for strong and weak biofilm producing respectively (figure 5). Microtiter plate is a useful method for studying biofilm formation in its early phases [13].

In this study the results showed that (90.6%) of P. mirabilis isolates has the ability to produce biofilm. This result were agree with the researcher finding [21, 22] who recorded that the biofilm formation was (90%, 94.3%) respectively. The bacterial ability to produce biofilm protected it from various stresses, involving antibacterial drugs and the immune responses. The ability to form biofilm has been related with recurrent chronic infections and raised antibiotic resistance, biofilm is a network of extracellular polymeric materials. The biofilms give the microorganism protective habitat so they can endure and resist antibiotic [23]

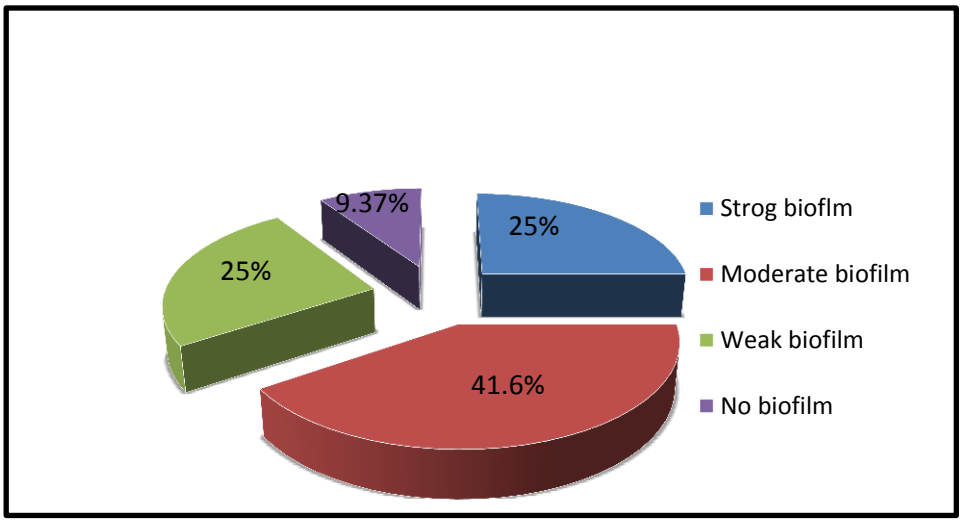


**Figure 6.** Biofilm production ratio in P. mirabilis

## The relation between biofilm production and antibiotic resistance

The results in table (3) show that there is a statistical relation between biofilm producer bacteria and non-biofilm producer for doxycycline antibiotic with a P value of 0.134 in which the result of resistance of biofilm producer for doxycycline is (93.1%) while for non-biofilm producer is (66.6%). Among the biofilm producer the most resistance antibiotic are doxycycline (93.1%), trimethoprim- sulfamethaxole (89.65%), ampicillin (72.4%), and amoxicillin /clavulante (68.96%). While the results of resistance for non-biofilm producers doxycycline (66.6%) and (33.3%) for each of trimethoprim- sulfamethaxole, ampicillin and amoxicillin. This study showed that biofilm producer are more resistance to antibiotics than non-biofilm producer agree with some of studies that showed there is a high relation between antibiotic resistance and biofilm production, and the production of biofilm increase the pathogenicity and difficulty in treatment, because of outer membrane lipoprotein and biofilms may play a significant role in bacterial resistance to antibiotics in contrast to other bacteria that lack biofilm [32, 33]. A sensitivity of (6.89%, 10.34%, 27.58%) were showed for the biofilm producing isolates to doxycycline, Trimethoprim-sulfamethaxole and Ampicillin respectively, while the non-producing biofilm showed sensitivity of (33.3%, 66.6%, 66.6%) doxycycline, Trimethoprim-sulfamethaxole and Ampicillin respectively, this indicate that the biofilm production plays an important role in making reducing sensitivity in biofilm producer compare with non-biofilm producer for their antibiotics [33].

**Table 3.** Relation between antibiotic resistance and *P. mirabilis* that biofilm producer and non-producer

| Antibiotic | Biofilm producer No. (29) | | | Non biofilm producer No.(3) | | | *P*.value |
|---|---|---|---|---|---|---|---|
| | Resistant | Intermediate | Sensitive | Resistant | Intermediate | Sensitive | |
| Amoxicillin/ clavulanic | 20(68.96%) | 0(0%) | 9 (31%) | 1(33.3%) | 0(0%) | 2(66.6%) | 0.216 |
| Ciprofloxacin | 7(24.13%) | 1(3.44%) | 21(72.41%) | 1(33.3%) | 0(0%) | 2(66.6%) | 0.726 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Doxycycline | 27(93.1%) | 0(0%) | 2(6.89%) | 2(66.6%) | 0(0%) | 1(33.3%) | 0.134 |
| Imipenem | 0 (0%) | 0 (0%) | 29(100%) | 0(0%) | 0(0%) | 3(100%) | 0.0 |
| Gentamycin | 11 (37.9%) | 2(6.89%) | 16(55%) | 1(33.3%) | 0(0%) | 2(66.6%) | 0.164 |
| Ampicillin | 21(72.4%) | 0(0%) | 8(27.58%) | 1(33.3%) | 0(0%) | 2(66.6%) | 0.164 |
| Trimethoprim-sulfamethaxole | 26(89.65%) | 0(0%) | 3(10.34%) | 1(33.3%) | 0(0%) | 2(66.6%) | 0.558 |
| cefotaxim | 12(34.8%) | 4(13.79%) | 13(44.8%) | 1(33.3%) | 0(0%) | 2(66.6%) | 0.787 |

## Conclusions

1. P. mirabilis is one of important pathogens which causing various types of infection, most commonly urinary tract infection.
2. P. mirabilis is highly resistant for many common antibiotics.
3. Clinical isolates of P. mirabilis ability to production of biofilm show moderate (41.6%), strong (25%) and weak (25%).
4. There is a relation between the production of biofilm and antibiotic resistance in P. mirabilis.

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