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Colistin resistance of non-pathogenic strains of *Escherichia coli* occurring as natural intestinal flora in broiler chickens treated and not treated with colistin sulphate

Michał Majewski[1]✉, Anna Łukomska[2], Jarosław Wilczyński[3], Danuta Wystalska[3], Przemysław Racewicz[1], Joanna Nowacka-Woszuk[4], Marcin Pszczola[4], Krzysztof Anusz[5]

[1]Laboratory of Veterinary Public Health Protection, Faculty of Veterinary Medicine and Animal Science, University of Life Sciences in Poznań, 62-002 Złotniki, Poland
[2]Department of Preclinical Sciences and Infectious Diseases, [4]Department of Genetics and Animal Breeding, Faculty of Veterinary Medicine and Animal Science, University of Life Sciences in Poznań, 60-637 Poznań, Poland
[3]Lab-Vet Laboratory, 62-080 Tarnowo Podgórne, Poland
[5]Department of Food Hygiene and Public Health Protection, Institute of Veterinary Medicine, Warsaw University of Life Sciences-SGGW, 02-787 Warsaw, Poland
michalm@up.poznan.pl

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Abstract

Introduction: A significant threat to public health is presented by antibiotic-resistant strains of bacteria, selective pressure on which results from antibiotic use. Colistin is an antibiotic commonly used in veterinary medicine, but also one of last resort in human medicine. Since the 2015 discovery in China of the *mcr-1* gene encoding colistin resistance in *Enterobacteriaceae*, other countries have noted its presence. This study was to find the *mcr-1* gene prevalence in *E. coli* isolated from poultry slaughtered in Poland. **Material and Methods:** Cloacal swabs were taken from December 2017 to October 2018 from broiler chickens in three regions. The samples (n = 158) were grouped as flocks treated with colistin sulphate (n = 87) and those not treated (n = 71). Resistance to antimicrobials commonly used in poultry was evaluated by minimum inhibitory concentration. The presence of the *mcr-1* gene was confirmed by PCR. **Results:** Isolates containing the *mcr-1* gene were yielded by 11.27% of the samples from not treated flocks and 19.54% of those from treated flocks, but no statistically significant difference in the prevalence of the gene was seen between the groups. **Conclusion:** The results clearly preclude intensification of selective pressure for colistin resistance due to colistin sulphate treatment because they show that the avian gastrointestinal tract was already inhabited by colistin-resistant *E. coli* by the time the chickens came to the poultry house.

Keywords: poultry, *Escherichia coli*, antibiotic resistance, colistin.

Introduction

The presence of antibiotic-resistant bacterial strains in farm animals used for food production presents a significant public health challenge. Since 2015, when the plasma-transferred *mcr-1* gene encoding for colistin resistance was discovered for the first time in China, there have been an increasing number of reports reflecting the rapid spread of resistance to this antibiotic (13). Livestock and food of animal origin are among the sources of microorganism transmission into the human body (12). Studies of *E. coli* resistance to colistin have usually involved strains isolated from pigs (3), but the bacteria inhabiting poultry digestive tracts can also gain resistance. Due to their short fattening period, the risk of bacterial infection in broiler chickens is lower than in other slaughter animals, but antibiotic therapy is frequently necessary during breeding. Chicken tissue is exposed to a risk of contamination and meat to a risk of cross-contamination much more frequently than the tissue and meat of other species. Contamination with

intestinal flora may occur at many stages, including during breeding, while transferring broilers to the slaughterhouse, or actually on those premises, given that the requirement for fast slaughter and post-slaughter processing deprioritises careful procedures and leads to contamination events being quite common. Intestinal injury can lead to the bacteria living in birds' digestive tracts being transferred to the surface of the meat, where it poses a threat to human consumers (17, 23).

In many cases, plasmids transmitting colistin resistance genes also transfer the genes for resistance to carbapenems and β-lactams with wide substrate spectrum (10, 19, 27). Moreover, the mechanism of colistin resistance can be conferred to other bacteria species through horizontal gene transfer. Reports published so far have described such occurrences in *Citrobacter braakii*, *Cronobacter sakazakii*, *Kluyvera ascorbata* and *Klebsiella aerogenes*, as well as in pathogens that pose serious threats to public health, such as *Salmonella enterica* and *Klebsiella pneumoniae* (25).

The most important pathogenic agents in which antibiotic resistance is known and which cause human gastrointestinal tract infections as a result of the consumption of undercooked poultry include *Salmonella* and *Campylobacter jejuni* (6, 11). The risk posed by antibiotic-resistant non-pathogenic strains of *E. coli* is much less often discussed, as these bacteria are less likely to cause food poisoning. Public health specialists have been paying increasing attention to the danger of transferring antibiotic resistance genes to other microorganisms. Mobile genetic elements, such as plasmids, are at the root of many such mechanisms. The *mcr-1* gene, discovered in 2015 in China encoding *E. coli* resistance to colistin, has been found in the plasmid pHNSHP45, which is classified as an IncI2 plasmid (13). Over the months following the gene's discovery, *mcr-1*-positive strains of *E. coli* were detected in animals and in some rare cases in humans, and were also isolated in other countries. The genes belonging to the *mcr* group were also localised on plasmids IncI2, IncHI2, and IncX4 (12, 15). The growing prevalence of colistin-resistant strains of *E. coli* is believed to originate from the overuse of colistin sulphate in animal husbandry. Colistin is commonly used globally to treat infections caused by *Enterobacteriaceae*, and as an antibiotic growth promoter added to feed. Since the increasing prevalence of colistin-resistant strains of *E. coli* has been detected in animals throughout the world, many governments that had previously taken a liberal approach to antibiotic growth promoter use have moved to ban this antibiotic as a feed additive (1, 12, 17, 24).

Colistin is a commonly used antibiotic in poultry production in Poland. This study evaluated the effects of medicinal preparations containing colistin sulphate on the incidence of the plasmid *mcr-1* gene in non-pathogenic strains of *E. coli* in the intestinal flora of healthy broiler chickens intended for slaughter.

Material and Methods

Study material and isolation of bacterial strain. The study material consisted of cloacal swabs taken from December 2017 to October 2018 from broiler chickens belonging to commercial poultry flocks from the three provinces of Wielkopolskie, Lubuskie, and Zachodniopomorskie. The swabs were collected in two slaughterhouses from dead birds that had previously been stunned in controlled atmosphere system (CAS) chambers and bled, as described in Regulation (EC) 1099/2009 (7). The samples came from flocks treated with colistin sulphate (n = 87) or not treated (n = 71). A total of 108 samples were collected in the first slaughterhouse (each sample was swabbed from five chickens chosen at random, from 70 treated and 38 not treated flocks), and 50 samples were taken in the second slaughterhouse (also in this instance each sample was swabbed from five chickens chosen at random, from 17 treated and 33 not treated flocks). The managers running these facilities agreed that the samples could be collected under the condition that no data identifying the slaughterhouses and farms be published. For this reason, only the regions in which the farms are located are given.

The samples were collected using NRS II Transwab swabs with 10 mL of buffered peptone water (Medical Wire & Equipment, Corsham, UK) from the cloaca of the slaughtered broilers. The material was plated on MacConkey Agar (Oxoid, Basingstoke, UK) supplemented with 2 mg/L of colistin sulphate (Sigma Aldrich, St. Louis, MO, USA). A stock solution was prepared by dissolving 20 mg of the standard in 5 mL of H_2O to a concentration of 4 mg/mL. The working solution was made by mixing the stock solution with water in a 1:1 ratio (2 mg/mL). The plates were incubated for 24 ± 2 h under aerobic conditions at 37°C ± 1°C. Following the incubation, a single colony with a phenotype typical of *E. coli* growing on MacConkey Agar was passaged to Nutrient Agar (Oxoid) to isolate a pure culture for further testing. The incubation conditions were 37°C ± 1°C and 24 ± 2 h.

Antimicrobial susceptibility testing by MIC. The minimum inhibitory concentrations (MICs) for antibiotics belonging to different chemical groups such as colistin, amoxicillin, amoxicillin/clavulanic acid, doxycycline, enrofloxacin, florfenicol, neomycin, norfloxacin, spectinomycin, and trimethoprim with sulphamethoxazole were assayed. The cultured colonies were suspended in demineralised water (Trek Diagnostic Systems, Cleveland, OH, USA) to obtain 0.5 McFarland turbidity, as assessed using a Sensititre Nephelometer (Trek Diagnostic Systems). *E. coli* suspension was spread with a sterile swab on Mueller–Hinton Agar (Oxoid) and the minimal bacterial growth

inhibitory concentration was determined using MIC Test Strip gradient strips (Liofilchem, Roseto degli Abruzzi, Italy). The incubation was conducted in an aerobic atmosphere at 35°C ± 1°C for 18–20 h. The results were confirmed using a microdilution method in a Vizion device (Trek Diagnostic Systems). To this end, colonies of the isolates were transferred to 4 mL of demineralised water (Trek Diagnostic Systems) to achieve a suspension of 0.5 turbidity on the McFarland scale. As previously, the turbidity was measured using a Sensititre Nephelometer (Trek Diagnostic Systems). The 10 µL of suspension thus prepared was transferred into 11 mL of Mueller–Hinton Broth (Trek Diagnostic Systems). The resulting inoculum was placed on a plate at 50 µL per well with a Sensititre AutoInoculator (both Trek Diagnostic Systems) and incubated at 37°C ± 1°C for 18–24 h. After incubation, the Sensititre plate was read in the Vizion device using SWIN software (Trek Diagnostic Systems). The MIC results were automatically interpreted by the SWIN software used by the equipment and reagent manufacturers based on FDA, CLSI and EUCAST guidelines.

To confirm MIC for colistin, an additional ComASP Colistin test based on the broth microdilution method (Liofilchem) was conducted, calibrated to MIC between 0.25 and 16 µg/mL. Control bacteria were analysed together with reference strains of *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *E. coli* NCTC 13846. The tests were repeated three times for each isolate. The bacterial suspension, with a density of 0.5 on the McFarland scale, was diluted 1:20 with saline solution. Then, 0.4 mL of the mixture was transferred to a test tube filled with Mueller–Hinton II Broth (Liofilchem). Each well of the assay plate was filled with 100 µL of the mixture. The plates were incubated at 36°C ± 2°C, and the results were read after 18 h.

DNA isolation. The isolated *E. coli* were stored at −80°C on Viabank Medium (Medical Wire & Equipment). To detect the possible presence of genes encoding colistin resistance, colistin-resistant isolates of *E. coli* were sieved onto Nutrient Agar (Oxoid) and held at 37°C ± 1°C for 24 ± 2 h. The control sample was a plasmid gene for *E. coli* strain ZTA14/01057 received from Visavet Health Surveillance Centre (Complutense University, Madrid, Spain). The negative control was provided by four isolates of *E. coli* not grown on a medium supplemented with colistin sulphate (2 µg/mL), and in which colistin resistance was not detected in a gradient diffusion test. The test was performed for a sample consisting of the living colony dissolved in 50 µL of distilled water, denatured for 5 min at 98°C and centrifuged at 10,000 rpm. The reaction consumed 3 µL of supernatant.

PCR conditions. After obtaining individual living colonies, bacterial DNA was examined using the PCR method. The reaction mixture of 20 µL volume contained: 2 µL of DNA, 0.3 µL of Taq (EurX, Gdańsk, Poland), and 1 µL of each starter: F – 5′-CGG TCAGTCCGTTTGTTC-3′ and R – 5′-CTTGGTCGG TCTGTAGGG-3′ (13). The PCR conditions were as follows: an initialisation step at 94°C for 5 min, 35 cycles of denaturation at 94°C for 40 s, annealing at 60°C for 40 s, and elongation at 72°C for 40 s, and final cooling of the product to 15°C. The reaction product contained 309 bp and was visualised in electrophoresis in 1.5% agarose gel. To assess the significance of the differences in the frequency of *mcr-1*-positive *E. coli* between samples from flocks treated and not treated with colistin sulphate, Pearson's chi-squared test was carried out on a contingency table, implemented in the chisq.test function in the base package of R statistical software (22).

Results

Phenotyping using the MIC Test Strip gradient strips and ComASP confirmed the resistance of 25 bacteria to colistin, with 4 µg/mL in 24 strains and 8 µg/mL in one strain.

Colistin-resistant strains of *E. coli* may be present in birds moved to a growing facility on the first day of their lives, or may be carried on contaminated equipment, as they were detected in different poultry houses on the same farm (five samples from different flocks kept in one location) within 47 days between the first and last sampling date. A similar situation occurred for the samples from four groups reared in two buildings at the same location. This means that 52% of the positive samples came from farms where at least two poultry houses accommodated broilers that carried the *mcr-1* gene.

All the isolated colistin-resistant *E. coli* showed multidrug resistance. All 25 *E. coli* isolates were resistant to amoxicillin, 28% (7/25) to amoxicillin/clavulanic acid, 24% (6/25) to doxycycline, 76% (19/25) to enrofloxacin, 64% (16/25) to norfloxacin, 40% (10/25) to florfenicol, 4% (1/25) to neomycin, 40% (10/25) to spectinomycin, and 80% (20/25) to trimethoprim with sulphomethoxazole. The results of multidrug resistance testing are shown in Table 1.

In addition, 60% (15/25) of *mcr-1*-positive isolates were intermediate susceptible to spectinomycin, 4% (1/25) to norfloxacin, 24% (6/25) to florfenicol, 52% (13/25) to doxycycline, and 28% (7/25) to amoxicillin with clavulanic acid.

The PCR results indicate the presence of the *mcr-1* gene in the 25 colistin-resistant strains (Fig. 1).

E. coli with the *mcr-1* gene plasmid was detected in samples from both slaughterhouses; its prevalence was 17.6% in the first slaughterhouse and 10% in the second one. Cloacal swabs from the broilers in slaughterhouse 1 revealed a significantly higher prevalence ($P = 0.02535$) of *mcr-1*-positive *E. coli* in the flocks treated with colistin sulphate (21.43%, 15/70) than in the not treated flocks (13.16%, 5/38). No

such difference (P=0.6547) was seen in slaughterhouse 2, where *mcr-1* prevalence was 9.09% in the treated flocks and 11.76% in the not treated flocks. The prevalence of *mcr-1*-positive strains in the samples from Wielkopolskie Province was 17.43% (19/109), while in those from Zachodniopomorskie Province it was 15.79% (6/38). The samples from Lubuskie Province contained no *mcr-1* gene, but there were few of these samples (0/11). In the flocks where the *mcr-1* gene was not detected, antibacterial therapy was applied an average of 3.06 times per flock, while in the *mcr-1*-positive flocks it was applied an average of 2.88 times per flock. Three multidrug-resistant isolates were sensitive only to neomycin and two were partially resistant to spectinomycin and florfenicol.

Table 1. Number of *E. coli* strains resistant to more than two antibiotics

Number of antibiotics:	Percentage of resistant bacteria (number of strains out of the total 25):
3	12% (3)
4	16% (4)
5	20% (5)
6	28% (7)
7	8% (2)
8	12% (3)
9	4% (1)

No significant difference was observed in the prevalence of the *mcr-1* gene between the flocks treated with colistin sulphate and those that were not treated (P = 0.07186). In the not treated flocks, *mcr-1*-positive E. coli was isolated in 11.27% of birds (8/71), while in the flocks treated with colistin this was found in 19.54% (17/87). All the results are presented in Table 2.

The farmers' declarations for the tested flocks showed that the most commonly used antibiotics were amoxicillin trihydrate (80%, 20/25), colistin sulphate (68%, 17/25), enrofloxacin (40%, 10/25), doxycycline (36%, 9/25), sulphamethoxazole with trimethoprim (36%, 9/25), lincomycin-spectinomycin (20%, 5/25), florfenicol (4%, 1/25), and amoxicillin with clavulanic acid (4%, 1/25).

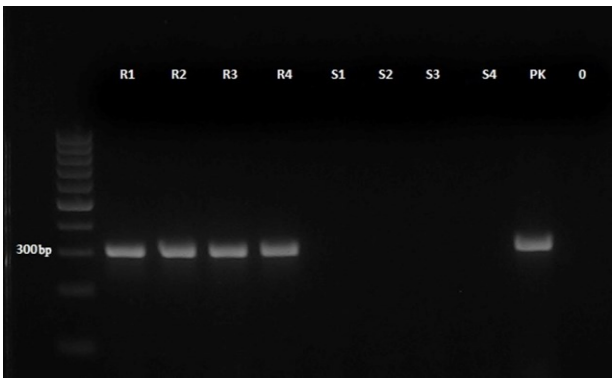

Fig. 1. Electrophoresis for *mcr-1* gene amplification of *E. coli* isolates with MIC exceeding 2 µg/mL. R1–4 – *mcr-1*-positive *E. coli*; PK – positive control; S1–4 – negative controls; 0 – sample without DNA

Discussion

Veterinary medicinal products are commonly used in broiler chickens to reduce losses caused by bacterial infections. Due to the prevalence of infections, antibiotics are the most common, and usually the only, type of pharmaceuticals used.

Only a few of the flocks investigated in this study were not treated over the six-week fattening period. The frequent use of medicines in poultry is a global problem, with different solutions being adopted to treat bacterial infections. In North America, the most commonly used group of drugs is the tetracyclines, which account for two-thirds of all antibiotics given to animals; in Europe, they account for 37% (2, 23). Our study showed that tetracyclines were rarely used to treat broilers (14.56% of flocks), even though data from the European Medicines Agency indicate that they accounted for 32.2% of all antibiotics sold in Poland (8). The most commonly used drugs were β-lactams (97% of flocks), fluoroquinolones (56% of flocks), and polymyxins in the form of colistin (55% of flocks). Colistin has been recognised as a critically important human antimicrobial by the WHO, so these data on the frequency of its veterinary use are disconcerting (26).

According to the European Medicines Agency, polymyxin (mg/population correction unit – PCU) sales in Poland increased by 35% between 2011 and 2016. In 2011 they made up 3.3% of all antibiotics, and in 2016 4.3%. Colistin is also used in farm animals in other European countries.

Table 2. Number of isolated colistin-resistant *E. coli* in the flocks treated with colistin sulphate and those not treated

	Number of flocks (n)	Percentage of flocks	Number of flocks carrying *mcr-1* gene (n)	Percentage of flocks carrying *mcr-1* gene
Not treated (a)	71	44.94%	8	11.27%
Treated (b)	87	55.06%	17	19.54%
Total	158	100.00%	25	15.82%

a – flocks not treated with colistin sulphate; b – flocks treated with colistin sulphate

It is particularly popular in Spain (22.00 mg/PCU), Italy (15.10 mg/PCU), Portugal (13.53 mg/PCU), Germany (7.89 mg/PCU), and Hungary (6.62 mg/PCU). Interestingly, despite its considerable use, polymyxin sales have dropped over the last seven years by 62% in Italy, 68% in France, and 47% in Germany. In this period, polymyxins were not used at all in Finland, Iceland, or Norway, and furthermore, countries where sales were already low, including the United Kingdom (<0.05 mg/PCU), Sweden (0.09 mg/PCU), Slovenia (0.1 mg/PCU), Latvia (0.89 mg/PCU), Lithuania (0.98 mg/PCU), and the Netherlands (0.31 mg/PCU), have striven to eliminate this antibiotic from the treatment of livestock (24). In other parts of the world, the use of pharmaceuticals in poultry is also frequent. Of 280 broiler flocks investigated from 2014 to 2016 in Morocco, 93% were treated for at least three days of the fattening period, usually with enrofloxacin, colistin, and trimethoprim. Colistin was used at 8.40 mg/kg (20).

The occurrence of antibiotic-resistant bacterial strains is an increasingly serious threat to both human medicine and animal production. The spread of antibiotic resistance among bacteria that inhabit the human body usually happens *via* their transmission from the environment. This may cause rapid escalation from single cases to a serious threat to public health, as in the case of *E. coli* ST131 or *K. pneumoniae* ST258 (16). *Mcr-1*-positive *E. coli* as isolated from the digestive tracts of farm animals has quickly spread all over the world and sometimes causes infections in humans. Colistin-resistant strains of *E. coli* also pose a threat in countries where antibiotic resistance is low. Finland is one of three European countries that does not use polymyxins in veterinary medicine, yet the country's first case of human infection with *E. coli* with the plasmid-transferred *mcr-1* gene has been reported (9, 12). *E. coli* strains with the plasmid *mcr-1* gene are most commonly isolated from farm animals, but they also pose a threat to public health, as evidenced by their isolation from other sources. Studies have revealed the highest average prevalence of pathogenic *E. coli* to be in animals (16.8%), followed by food (7.1%) and humans (0.7%) (5). Moreover, the first reports have been published of colistin-resistant *E. coli* producing extended spectrum beta-lactamase being isolated from the environment (29). MIC values for these isolates ranged from 4 to 8 µg/mL and were consistent with data from another study that investigated the resistance of pathogenic *E. coli* from chicken organs to colistin. Of the 13 *mcr-1*-positive strains isolated between 2004 and 2012 by Yassin *et al.* (28), 11 (84.6%) showed resistance to colistin at 4 µg/mL, while the other 2 (15.4%) were resistant at 8 µg/mL. However, the prevalence of colistin-resistant pathogenic *E. coli* was lower than in our study, being in only 3.2% (13/404) of the samples.

The risk exists of colistin-resistant *E. coli* strains in the broiler intestinal tract being transferred onto the surface of the meat as the carcass is processed. Monte *et al.* (17), who investigated fresh poultry meat sold in south-east Brazil, confirmed colistin resistance in 8 out of 41 samples (19.5%). Apart from the *mcr-1* gene, the isolated strains of *E. coli* also included the $bla_{CTX\text{-}M}$ and $bla_{CMY\text{-}2}$ genes encoding resistance to β-lactam antibiotics. This means that poultry meat can be considered a potential source of genes of colistin resistance (17). A study published by Nguyen *et al.* (18), which evaluated the prevalence of the *mcr-1* gene in broilers and pigs in southern Vietnam, detected the presence of colistin resistance genes in respectively 22.2% and 24.4% of *E. coli* isolated from the final segment of the digestive tract. The authors found no changes in the prevalence of *mcr-1* genes during the production cycle. The median MIC was 4 µg/mL (18).

The presence of antibiotic-resistant bacteria is one of the key issues affecting poultry antimicrobial chemotherapy. Literature data confirm that such microorganisms are more prevalent in treated flocks than in those that have not been treated. Dheilly *et al.* (4) demonstrated the increased prevalence of multiresistant bacterial strains in birds treated with oxytetracycline, trimethoprim with sulphadiamethoxine, amoxicillin, or enrofloxacin. The growth of a tetracycline-resistant *E. coli* population has been reported only in birds receiving oxytetracycline, whereas multiresistant *E. coli* strains were detected in *E. coli* populations isolated from birds treated with trimethoprim with sulphadimethoxine, amoxicillin, and enrofloxacin (4). For the same geographical area, *mcr-1*-positive *E. coli* strains isolated from broilers in the years 2014–2016 showed greater resistance to amoxicillin than pathogenic *E. coli* strains (100% in the experimental group *vs*. 73.64%). A similar situation was observed for amoxicillin with clavulanic acid (28% *vs*. 17.20%), enrofloxacin (76% *vs.* 48.65%), norfloxacin (64% *vs.* 45.06%), and trimethoprim with sulphamethoxazole (80% *vs.* 46.42%).

A lower prevalence of doxycycline and neomycin insusceptibility was observed in resistant strains: 24% in the experimental group *vs.* 52.08% and 4% *vs.* 19.33%, respectively. Similar data across experimental and control chicken groups were obtained for spectinomycin and florfenicol, where the values were 40% in the experimental group *vs.* 39.087% and 40% *vs.* 38.15%, respectively. A total of 60% of the isolates in both groups were classified as moderately susceptible to spectinomycin, 4% in the experimental group *vs.* 3.83% to norfloxacin, 24% *vs*. 44.16% to florfenicol, 52% *vs.* 11.89% to doxycycline, and 28% *vs.* 9.75% to amoxicillin with clavulanic acid (14).

Antibiotics and chemotherapeutics will undoubtedly remain the main tools in fighting poultry infections in the coming years, while researchers continue to seek alternative treatment methods. Finding truly effective substitutes poses a considerable challenge for researchers investigating treatment methods that would be useful not only for poultry, but

also for other animals, and particularly for humans. Searching for alternative treatments and creating conditions conducive to reducing the incidence of infection are the greatest imperatives to address if we are to maintain the efficacy of antibiotics. Considering that multidrug resistant strains are also present in sporadically treated flocks, we should assume that multiresistant bacteria colonise the digestive tract of birds before they are placed in the growing house.

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