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Phenotypic and Molecular Detection of β-lactamase Genes bla_{TEM}, bla_{CTX}, and bla_{SHV} Produced by *Salmonella* spp. Isolated from Poultry Meat

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Abstract

Background: One of the most important and frequently encountered pathogens in the poultry supply chain is *Salmonella*. A comprehensive insight into its pathology and its counteractive mechanisms towards antibiotics, thus, is much needed. The determination of phenotype and molecular susceptibility along with the detection of β-lactamase genes produced by *Salmonella* isolated from poultry meat was the objective of the present study.

Objectives: Additionally, this study is significant due to the prevalence of *Salmonella* in Ardabil, a city in the northwest of Iran.

Methods: The present study aimed to analyze the susceptibility of *Salmonella* isolates (collected from the commercial broilers (CB) and spent hens (SH)) towards the β-lactam class of wide-spectrum antibiotics. Further, the phenotypes of the isolates were investigated based on their extended-spectrum β-lactamase (ESBL) production. The test samples were collected from a total of 100 chicken carcasses comprising 50 from the retail CB and 50 from the SH procured from a local supermarket in Ardabil city. The *Salmonella* isolates were then analyzed for the production of β-lactamase enzymes under the standard laboratory conditions.

Results: 20 *Salmonella* strains were isolated from the 100 samples, with 55% (11/20) from the CB and the remaining 45% (9/20) from the SH. The isolated *Salmonella* spp. showed multiple β-lactam resistance phenotypes and the presence of β-lactamase genes. bla_{TEM} was found to be the dominant β-lactamase gene (85%), followed by $bla_{CTX\text{-}M}$ (60%) and bla_{SHV} (35%). Using clinical and laboratory standards institute extended-spectrum β-lactamase (CLSI ESBL) confirmatory test, 100% of the isolates were found to be ESBL producers, as also confirmed by the PCR method.

Conclusions: This study revealed that a significant number of antibiotic-resistant *Salmonella* was isolated from the retail poultry meat samples of the CB and SH. Mortality and morbidity rates increase with the increase in the resistance of bacteria to standard antibiotics. Therefore, microbiological surveillance for different isolates of *Salmonella* should be done at the country level to monitor its antimicrobial resistance. The prime purpose of the current study was to centralize the focus in this regard, via two advanced detection procedures comprising phenotypic and molecular detection of β-lactamases enzymes produced by *Salmonella* isolates from poultry meat.

Keywords: *Salmonella*, β-lactamase, Poultry Meat, Extended-Spectrum β-lactamase, Antibiotic Resistance

1. Background

Salmonellae are non-sporulating, flagellated, Gram-negative, facultative anaerobic bacilli belonging to the Enterobacteriaceae family containing more than 2,300 serotypes. Antibiotics such as ampicillin, chloramphenicol, and trimethoprim/sulfamethoxazole have been used for the treatment of enteric fever for many years. Later in the 1980s, the resistance of *Salmonellae* towards multiple drugs was reported. This caused the increasing use of fluoroquinolone alternatives such as ciprofloxacin and extended-spectrum cephalosporins (1). Recently, azithromycin has also been reported to be effective in the treatment of enteric fever (2, 3).

The literature highlights the production of extended-spectrum β-lactamase (ESBL) in *Salmonella typhi*, which is directly related to their antibiotic resistance. This resulted in the failure of treatment in many countries where cefotaxime, ceftriaxone, and ciprofloxacin were used as antibiotics (4).

The ESBLs reported in *Salmonella* spp. include *TEM* (*Temoneira*), *SHV* (containing sulfhydryl variable active sites), and *CTX-M* (cefotaximase hydrolyzing activity) (5).

The most commonly found ESBLs in Enterobacteriaceae, including *Salmonellae*, are *CTX-M*. In 1989, the earliest CTX-M1 type of cefotaximase was isolated in Germany (6). Around 300 different types of ESBLs have been reported (7), with TEM and SHV being the most commonly occurring variants (8). Nevertheless, over the past decade, several countries have witnessed a rise in non-TEM, non-SHV variants of ESBLs such as CTX-M (8). ESBL generation is mainly linked to mutations in β-lactamase enzymes encoded by bla_{SHV}, bla_{TEM}, and $bla_{\text{CTX-M}}$ genes.

2. Objectives

The present study aimed to document the existing dominance of ESBL-producing *Salmonella* spp. found in poultry meat from the commercial broiler (CB) and spent hens (SH) in Ardabil, located in the northwest of Iran.

3. Methods

This study was conducted in the Department of Microbiology of Ardabil University. The following materials and methods were used:

3.1. Bacterial Isolation

Overall, 100 retail chicken carcasses (comprising 50 from the CB and 50 from the SH) were used to collect test meat samples. The samples were taken from the CB and SH of local supermarkets and wet markets of Ardabil city. In total, 20 *Salmonella* strains were isolated from these collected meat samples. To this end, 25 g of each sample was transferred to 225 mL of the pre-enrichment medium consisting of peptone water and placed in an incubator at 37°C for 24 hours. The broth was then added to the selenite-F environment and incubated for 24 hours. The selenite-F medium was then inoculated into Salmonella-Shigella agar and brilliant green agar media and incubated for 24 hours at 37°C. Colorless colonies with black centers appeared in Salmonella-Shigella agar and yellow-colored colonies with black centers were visible in the brilliant green agar. These were suspected to be the colonies of *Salmonella* and were identified by using several biochemical tests such as urea, IMVIC, and TSI. Isolates enriched in selenite-F broth were carefully streaked on Salmonella-Shigella agar plates and incubated. Pure colonies were selected from the agar plates and then identified with standard bacteriological techniques.

3.2. Serotype Identification

The Kauffmann-White scheme was used for the identification of *Salmonella* serotype based on surface antigens. Serotyping was performed after the identification of species on a fresh pure culture of *Salmonella*. The antisera of *Salmonella* were purchased from Padtan-Teb Company. With the help of polyvalent antisera, the agglutination test was performed. The determination of serogroup was done by the agglutination on a slide with O-somatic antiserum. Then, specific monovalent antisera were used and allowed to stand on it for 10 seconds so that the antigen could be identified. The primary culture was treated with saline and then with O-antigen. The type of O-antigen was determined by oligosaccharides associated with outer membrane lipopolysaccharides. If agglutination occurred, it indicated that the serotype had been treated with a specific antiserum. After this, the serotype was treated with H-antigen (phase 1 and phase 2). The type of H-antigen was determined by the flagellar protein of bacteria. Based on the agglutination pattern obtained after this procedure, the serotype was identified by the Kauffmann-White reference catalog as B or D. In the Kauffmann-White (KW) scheme, the antigenic properties and variations of the O (surface polysaccharide) and H (flagellar) antigens from each serovar are described, which are known as the antigenic formulae.

3.3. Antimicrobial Susceptibility

Using the disk diffusion technique, the susceptibility of *Salmonella* towards antibiotics was determined on Mueller-Hinton agar plates. Interpretations were made in accordance with the guidelines of CLSI 2015 (9). The antibiotics tested were amoxicillin (30 μg), ampicillin (10 μg), cefotaxime (30 μg), ciprofloxacin (5 μg), nalidixic acid (10 μg), spectinomycin (10 μg), tetracycline (30 μg), and sulfamethoxazole (1.25/23.75 μg). The minimum inhibitory concentration (MIC) of cefotaxime and ceftriaxone was determined by the agar dilution method with the tested range of 0.008 - 128 μg/mL and then interpreted in accordance with the CLSI 2015 guidelines (9). In order to determine the MIC, different intended concentrations of each antibiotic were inoculated by using a fixed volume of nutrient broth containing a standard concentration of bacteria (0.5 McFarland) and then, the suspensions were incubated and examined for turbidity. A turbid sample indicated bacterial growth whereas a clear sample indicated the inhibition of bacterial growth and the corresponding concentration of the antibiotic used was considered as the MIC.

3.4. Screening for ESBL Production

The screening of ESBL production in *Salmonella* isolates was performed by using the inhibition zone test in accordance with the NCCLS recommendations. Discs of ceftazidime (30 μg) and cefotaxime (30 μg) were used for this study.

3.5. Phenotypic Confirmatory Test for ESBL Production

Mueller-Hinton agar culture medium was used to perform this study. Four different antibiotic discs were used, which included cefotaxime (30 μg), cefotaxime/clavulanic acid (30 μg/10 μg), ceftazidime (30 μg), and ceftazidime/clavulanic acid (30 μg/10 μg). The study was performed in accordance with the NCCLS recommendations.

3.6. Polymerase Chain Reaction (PCR) Amplification

The bacterial DNA was extracted by using the boiling method (10, 11). From tryptic soy broth (TSB), *Salmonella* strains were reactivated on tryptic soy agar (TSA) upon incubation for 18 - 24 hours. Later, they were inoculated in Luria Bertani broth (LB broth, 2 mL). After incubation for 18 - 24 hours, the LB broth was centrifuged (10,000 rpm for 10 minutes). The bacterial cell pellet collected was re-suspended in 500 μL of phosphate buffer (100 mM, pH 7) to enfeeble the cell membranes. Upon immersion of re-suspended bacterial cells in a boiling water bath (100°C) for 15 minutes, the membranes ruptured to release the genetic material. The solution containing the genetic material was collected and the DNA content was precipitated with 250 μL of absolute alcohol; then, it was washed twice with 1000 μL of 70% alcohol (stored at -20°C), dried, and re-suspended in 100 μL of sterile water.

This method was used for preparing lysates for all the isolates. All the isolates were tested positive for ESBLs production phenotypically. They were later screened for the presence of bla_{CTX}, bla_{SHV}, and bla_{TEM} genes by PCR assay using specific primers listed in Table 1. The PCR assay was carried out in 0.2-mL thin wall tubes using each of the bacterial lysates as template DNA. Each tube consisted of 1.5 mM $MgCl_2$, 0.2 μM of each primer, 200 μM of each dNTPs, 1.5 U of Taq polymerase (CinnaGen, Tehran, Iran), and 2.0 μL DNA template, making up overall 25 μL as the final volume. The PCR assay was performed in a thermal cycler and the cycling condition for $bla_{\text{CTX-M-1}}$ and bla_{SHV} was maintained as follows: (I) Initial denaturation at 94°C for seven minutes; (II) 30 cycles of amplification with denaturation at 94°C for 30 seconds; (III) annealing at 57°C for 30 seconds and an extension at 72°C for 30 seconds; and (IV) a final extension at 72°C for five minutes. For the bla_{TEM} gene, the annealing temperature was maintained at 53°C. The multiplex PCR assay was performed using the same composition of the PCR mixture described earlier, with an annealing temperature of 54°C.

4. Results

20 *Salmonella* strains were isolated from 100 poultry meat samples collected. 55% of the isolates (11/20) were obtained from CB meat and the remaining 45% (9/20) from SH meat. The isolated *Salmonella* serogroups were identified as B (five isolates) or D (15 isolates). The results are provided in Table 2. The antibiotic susceptibility test showed a significant amount of antibiotic resistance. The phenotypic confirmatory test was used for the detection of β-lactamases enzymes. An increase of $>$ 5 mm in the inhibition zone diameter was observed for all the antimicrobials when used in combination with clavulanic acid compared to when used alone. This is possibly due to the pronounced effect of ESBL on the individual antimicrobials compared to their combination with clavulanic acid. The size of the inhibition zone was considered as a marker indicating the presence or absence of ESBL in pre-screening. The positive indication for the presence of ESBL was given by the inhibition zone diameters obtained from the antibiotics, i.e. the inhibition zone diameter of $\leq$ 22 mm for ceftazidime and $\leq$ 27 mm for cefotaxime, further analyzed for the ESBL presence by the ESBL confirmatory test.

The results of the initial screening test were in accordance with the results obtained from the complementary confirmatory test of ESBL, with more than 90% of the isolates being positive in both analytical tests. The negative results of the screening test were also in agreement with the confirmatory test results, with some exceptions. For a few *Salmonella* isolates, although a positive outcome was obtained in the screening test, the confirmatory test result for ESBL production was found to be negative. Thus, the overall results indicated that the routine susceptibility test is a relatively less sensitive technique, failing to identify ESBL production in 25% of B (*Salmonella typhimorium*) and 75% of D (*Salmonella enteritidis*) isolates. The results are summarized in Table 2.

In fact, the results obtained from the routine susceptibility test showed that *Salmonella* strains belonging to these two serotypes exhibited antibiotic susceptibility towards cefotaxime and ceftazidime to some extent. About 78.7% - 94.0% of the total isolates that were cephalosporin-resistant were found to produce ESBL. The results are

Table 1. The Pattern of bla_{TEM}, bla_{SHV}, and bla_{CTX} Genes

Gene	Primer Sequence	Product Size	Reference
***bla*$_{CTX}$**		950	Schmitt et al. (12)
Forward	5-CCCATGGTTAAAAAACACTGC-3		
Reverse	5-CAGCGCTTTTGCCGTCTAAG-3		
***bla*$_{TEM}$**		1080	Weill et al. (13)
Forward	5-ATAAAATTCTTGAAGACGAAA-3		
Reverse	5-GACAGTTACCAATGCTTAATC-3		
***bla*$_{SHV}$**		747	Sun et al. (14)
Forward	5-ATGCGTTATATTCHCCTGTG-3		
Reverse	5-TGCTTTGTTCCGGGCCAAAC-3		

Table 2. The Results of Serotype Identification and ESBL Production Obtained from the Phenotypic Confirmatory Test (Total No. of Isolates, N = 20).

Serotypes	B (*Salmonella typhimurium*)	D (*Salmonella enteritidis*)
No. of isolates	5	15
ESBL producers, %	25	75

Table 3. Susceptibility and Resistance of Different Antibiotics (in Percentages) Against *Salmonella* spp.

S. No.	Antibiotics	Susceptibility, %	Resistance, %
1	Ampicillin	5	90
2	Amoxicillin	0	100
3	Cefotaxime	5	95
4	Ciprofloxacin	5	95
5	Nalidixic acid	5	95
6	Spectinomycin	0	100
7	Tetracycline	5	85
8	Sulfamethoxazole	15	70

Table 4. The Total Number of Genes Amplified During PCR (Total Number of Isolates, N = 20)

Genes	No. of Isolates (Frequency%)
TEM	17 (85)
CTX-M	12 (60)
SHV	7 (35)

graphically represented in Figure 1 where the percentage of *Salmonella* isolates (out of 20 isolates) exhibiting susceptibility or resistance towards each antibiotic has been plotted. The analysis of antimicrobial-resistance showed that resistance to β-lactam agents was more frequent in ESBL-producing isolates (Figure 1).

Figure 1 clearly shows that, in general, *Salmonella* spp. isolates are resistant towards most of the antibiotics. The isolated samples of *Salmonella* from both CB and SH showed the significant presence of both β-lactamases enzymes. Moreover, all the *Salmonella* isolates showed a high antibiotic resistance to amoxicillin (100%), spectinomycin (100%), cefotaxime (95%), nalidixic acid (95%), and ciprofloxacin (95%). The lowest resistance was shown to tetracycline (70%) as observed in Table 3.

The results of PCR amplification of bla_{TEM}, bla_{CTX-M}, and bla_{SHV} genes in *Salmonella* isolates from the CB and SH are given in Figure 2.

bla_{TEM} was the dominant β-lactamase gene (85%), followed by bla_{CTX} (60%) and bla_{SHV} (35%) (Table 4). The accession numbers JQ735915 for the β-lactamase bla_{TEM} gene, JN003854 for the bla_{SHV} gene, and EF592571 for the bla_{CTX-M} gene from GenBank were used. In addition to the study for the detection of β-lactamases, it was also revealed that a significant number of antibiotic-resistant *Salmonella* were presently isolated from the retail poultry meat samples of CB and SH.

5. Discussion

Enteric fever is endemic due to *Salmonella enterica*. The pathogens are transmitted through the fecal-oral route due to the lack of hygiene. Antibiotic therapy constitutes the mainstay of the management of enteric fever. The purpose of the current study was to detect β-lactamase enzymes produced by *Salmonella* isolated from poultry meat through two advanced detection procedures comprising

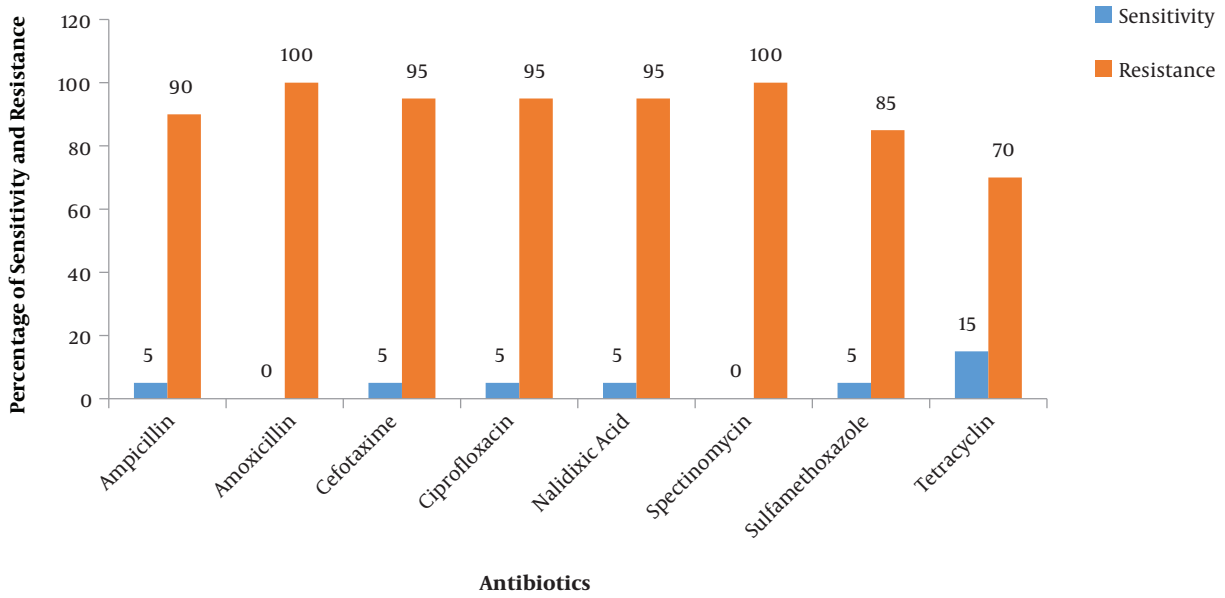

Figure 1. Percentage of *Salmonella* isolates (total of 20 isolates) exhibiting susceptibility or resistance towards each of the following antibiotics: amoxicillin (30 μg), ampicillin (10 μg), cefotaxime (30 μg), ciprofloxacin (5 μg), nalidixic acid (10 μg), spectinomycin (10 μg), tetracycline (30 μg), and sulfamethoxazole (1.25/23.75 μg).

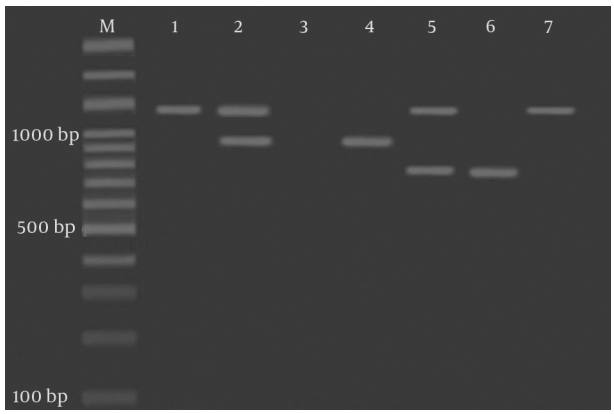

Figure 2. Agarose gel electrophoresis of the PCR amplified products. M: Molecular ladder; 1: bla_{TEM} positive control at 1080 bp; 2: *Salmonella* isolates showing bands at 1080 bp and 950 bp; 3: Negative control; 4: bla_{CTX} positive control at 950 bp; 5: *Salmonella* isolates showing bands at 1080 bp and 747 bp; 6: bla_{SHV} positive control at 747 bp; and 7: *Salmonella* isolates showing a band at 1080 bp only.

phenotypic and molecular detection. 20 *Salmonella* isolates were obtained from 100 meat samples, with 11 isolates from the CB and nine isolates from the SH. The serotypes identified were B (*Salmonella typhimurium*, five isolates) and D (*Salmonella enteritidis*, 15 isolates). The phenotypic detection of metallo-β-lactamase was done in 11 (100%) CB and nine (100%) SH samples.

In the present study, the susceptibility of *Salmonella* was studied against antibiotics such as amoxicillin (30 μg), ampicillin (10 μg), cefotaxime (30 μg), ciprofloxacin (5 μg), nalidixic acid (10 μg), spectinomycin (10 μg), tetracycline (30 μg), and sulfamethoxazole (1.25/23.75 μg). *Salmonella* spp. isolates were found to be resistant towards most of the antibiotics, with very less susceptibility to tetracycline and ampicillin. In a similar study, Bradford (15) reported the least antibiotic resistance of *Salmonella* isolates towards aztreonam (10%), followed by ceftazidime (14%), ceftriaxone (21%), cefoxitin (23%), and cefotaxime (32%). In addition, ESBL producers offered higher antibiotic resistance compared to non-ESBL producers. A relationship was observed in *Salmonella* spp. between ESBL production and antibiotic resistance towards ceftazidime and ceftriaxone. This reinforces the recent finding that most ESBLs display a special affinity to degrade ceftazidime (16). In this study, the 20 isolates obtained from the CB and SH were analyzed using PCR assay for the simultaneous detection of bla_{SHV} 747 bp, bla_{TEM} 950 bp, and bla_{CTX} 1080 bp. Ehlers et al. (16) found that M-PCR simultaneously amplified and detected the presence of bla_{SHV} (747 bp), bla_{CTX-M} (593 bp), and bla_{TEM} (445 bp). In a study carried out by Qiao et al. (17), it was found that 57.3% of the isolates harbored bla_{TEM} whereas 30.2%, 24.0%, 18.8%, and 7.3% of the isolates carried bla_{OXA-1}, $bla_{CTX-M-15}$, $bla_{CTX-M-3}$, and bla_{PSE-1} genes, respectively. In our study, the comparatively higher values of 85%, 60%, and 35% were found for *TEM*, *SHV*, and *CTX-M* genes, respec-

tively. These results are consistent with the findings reported from Tunisia where 86.44% of ESBL producing *Klebsiella* spp. were isolated (18). These findings show the importance of detection of resistant genes to ensure the appropriate treatment and use of proper control measures against such infections and their causative agents.

The underlying mechanism of β-lactam antibiotic resistance is through the production of β-lactamases. These enzymes function by hydrolyzing the β-lactam ring by breaking the amide linkage, thereby disabling their capability of inhibiting bacterial cell wall synthesis (19-21). Previously, it was known that ESBL-producing bacteria thrived commonly in hospital and clinical settings where extensive use of antimicrobial drugs and agents assisted in the development of their antimicrobial resistance (22). However, in the present study, we report the isolation and identification of ESBL isolates from poultry meat from broiler production chain. This study has a serious implication and intends to draw attention to the fact such pathogenic microorganisms can easily find a way into the human food chain (23). Moreover, such pathogens not only can spread from infected chicken to all other meat products, but also can contaminate an entire slaughter line triggering an outbreak of food poisoning (24-27).

The emergence of cheap generic alternatives has allowed the continued usage of prohibited drugs and additives in poultry farming as common measures to prevent infection. In addition, the extensive use of prescribed β-lactam antibiotics to treat *Salmonella* infections has contributed to a rise in antibiotic-resistant microorganisms (28, 29).

5.1. Conclusions

In this study, the isolated *Salmonella* were identified as B (five isolates) or D (15 isolates) serotypes. The ESBL screening study indicated the presence of ESBL in isolates from both CB and SH. Some of the *Salmonella* serotypes examined in this study showed resistance to multiple β-lactam antibiotics. An increase in the inhibition zone diameter was observed for all the antimicrobials when used in combination with clavulanic acid compared to when used alone. This is possibly due to the pronounced effect of ESBL on the individual antimicrobials compared to their combination with clavulanic acid. This indicates the scope of combinatorial antibiotic therapy in treating infections caused by *Salmonella*.

Footnotes

Authors' Contribution: My authorship statement is be based on the following: Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; and drafting the work or revising it critically for important intellectual content; and final approval of the version to be published; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of Interests: There is no conflict of interest.

Ethical Considerations: Not applicable (no human or animal experimentation was used).

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