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## Evaluation of the bacteriological flora in the vagina of postpartum anestrus cattle before and after treatment with three progesterone implants

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### Abstract

Thirty postpartum (>70 days) anestrus crossbred cows were treated with three standard hormonal protocols (CIDR TRIU-B, and P<sub>4</sub> Sponge n=10 each) for induction of estrus and the findings were compared with a group of untreated anestrus cows (Blank Sponge n=10). 80 vaginal swabs were collected aseptically from each group just before insertion of Implants/Blank Sponge and at the time of Implant/Blank Sponge withdrawal for microbiological evaluation of vaginal mucosa. Total number of isolates obtained from all the swabs were 180. Out of 180 isolates, 72 (40.00%) isolates were Gram positive and 108 isolates were (60.00%) Gram-negative bacteria, indicating dominance of Gram-negative bacteria. Overall ratio of number of isolates per vaginal sample obtained in the present study was 2.25. The highest rate of isolation of bacteria per sample was recorded in the placebo group at the time of removal of placebo i.e. 2.6, followed by 2.5 by same group at the time of insertion, 2.3 at the time of TRIU-B removal, 2.2 at the time of TRIU-B insertion and P<sub>4</sub> Sponge removal, 2.1 in CIDR at the time of removal and P<sub>4</sub> Sponge insertion and lowest i.e. 2.0 in CIDR group at the time of insertion of implant. The frequency of single and mixed isolates observed in different groups of anestrus animals at the time of insertion and removal of implants showed predominance of mixed isolates over single isolates 63 (78.75%) vs 17 (21.25%), indicating dominance of mixed isolates over single isolates. *E. coli*, *Staphylococcus*, *Proteus* and *Bacillus* were the commonest isolates obtained before and after insertion of implants and Blank Sponge in post-partum anestrus cattle. The implant used and time/period of isolation did not alter the types of bacteria isolated.

**Keywords:** Cow vaginal microflora, anestrus, estrus induction, progesterone implants

### 1. Introduction

The postpartum anoestrus in cattle has been identified as one of the prevalent problems affecting the reproductive efficiency which is in turn a major source of economic loss to dairy farmers in rural areas. Since the early work done by Robinson (1967)<sup>[29]</sup>, intravaginal inserts/sponges impregnated with progesterone are commonly administered to postpartum dairy cows for the treatment of anoestrus and provide a very convenient method of delivering this steroid hormone for prolonged periods (Rhodes *et al.*, 1998)<sup>[28]</sup>. Under natural conditions, the environment of the bovine vagina is stable and does not allow excessive growth of pathogenic, potentially pathogenic or saprophytic organisms (Hafez and Hafez, 2002 and Otero *et al.*, 2000)<sup>[15, 22]</sup>. Vaginal microorganisms can invade the uterus through the cervix, which is partly open due to the action of estrogens released during estrus or during parturition (Foldi *et al.*, 2006)<sup>[11]</sup>. Thus, the presence of microorganisms in the cervico vaginal secretions poses a potential threat to fertility of the cows (Dabas *et al.*, 1995)<sup>[7]</sup>. There is a lack of information about the effects of progestagen impregnated sponges/implants on the bacterial microflora in the vaginal microbiota. Therefore, the purpose of the current experiment was to conduct the qualitative analysis of bacteria from vagina associated with the use of different intravaginal implants in cows subjected to estrus induction protocols.

### 2. Materials and methods

The present study was carried on 40 anestrus cattle with more than 70 days postpartum period. The animals were selected from various villages of Jammu and Samba districts of J& K. The animals were randomly allocated to four groups, three treatment (n=10) and one untreated anestrus control (n=10) and were managed with three different hormonal regimens. In 10 true anestrus cattle, CIDR was inserted intra-vaginally on day 0,

removed on day 9 together with 600 IU i/m injection of PMSG/eCG and AI was performed on day 11 and 12 at detected heat. Same protocol as that of for CIDR group was followed in TRIU-B and P<sub>4</sub> Sponge group while as in control group blank sponge was inserted intra-vaginally on day 0 and was removed on day 9 together with injection of normal saline (10 ml) intramuscularly.

### 2.1 Collection of vaginal swab samples

80 vaginal swabs were collected on day 0 (insertion of implants) and day 9 (removal of implants). The vulvar area

was washed with water followed by surgical spirit and then a sterile pressed cotton swabs (1cm<sup>3</sup>), moistened with saline (0.9% NaCl; w/v) swab was inserted along the dorsal vaginal wall to approximately 10 cm cranial to the vulva, kept there for at least 30 seconds (Fig. 1). Upon collection, samples were placed individually into sterile tubes containing 10 ml of saline solution, which were kept in ice until arrival at the Public Health Laboratory at Faculty of Veterinary Sciences and Animal Husbandry SKUAST J, R.S. Pura, Jammu.

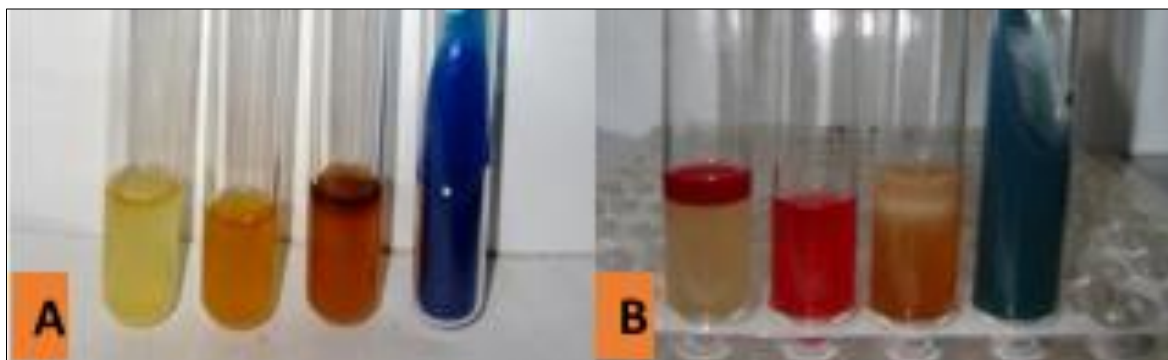


**Fig 1:** (A) Perineal region following cleaning with water followed by surgical spirit. (B) Moistened Cotton Swab inserted along the dorsal vaginal wall to approximately 10 cm cranial to the vulva.

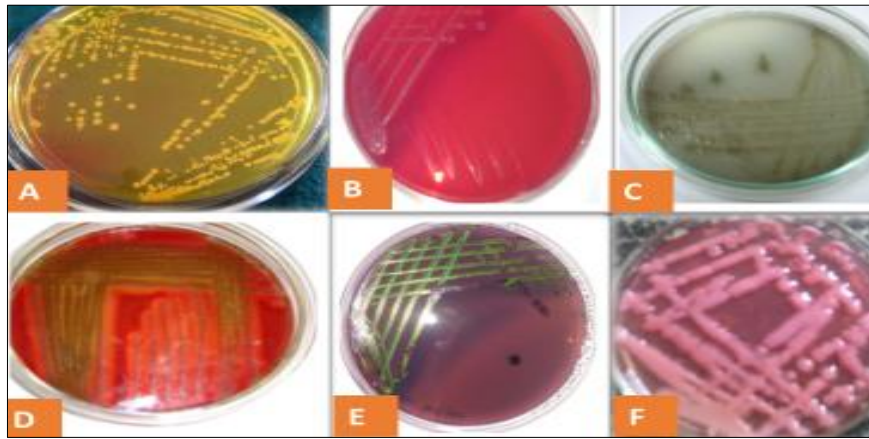
### 2.2 Bacterial isolation and identification

The vaginal swabs were cultured using different media for isolation and purification of bacteria which included: Blood agar, MacConkey agar, Nutrient agar, Edward blood agar, Eosin-Methylene-Blue agar (EMB), Simmon's citrate agar and Mannitol salt agar. The commercial media were prepared according to the direction of the manufacturer (Hi-Media, India). Plates were incubated at 37°C, and examined daily for

bacterial growth, for a period of 3 days for bacterial isolation (Quinn *et al.*, 2011<sup>[26]</sup>). All bacterial genera isolated were identified on the basis of colony characteristics, Gram staining, and biochemical reactions, including: urease test; citrate test; indole test, motility test, nitrate test, catalase activity tests, the fermentation of carbohydrates; and triple sugar iron (TSI) (Fig. 2) Genera were classified according to Cruickshank *et al.* (1980)<sup>[6]</sup> (Fig. 3).



**Fig. 2:** IMViC tests for identification of bacteria: (A) IMViC tests for *Pseudomonas* (-, -, -, +); (B) IMViC tests for *E. coli* (+, +, -, -)

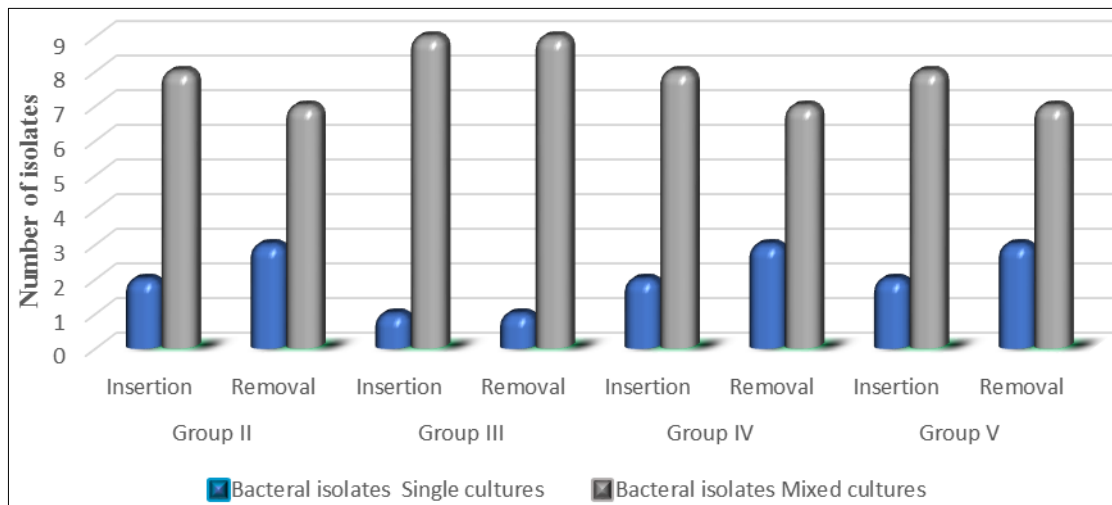


**Fig 3:** Selective Media for Identification of bacteria: (A) Growth of *S. aureus* on Mannitol Salt Aggar; (B) Pink colour colonies of *E. coli* on Mac Conky Agar; (C) Green colour discoloration of nutrient agar by *Pseudomonas* spp; (D) *Streptococcus* spp. on Blood agar with Haemolysis; (E) Metallic sheen appearance of *E. coli* on EMB (F) Growth of *Klebsiella* spp. on MacConkey agar.

**3. Results**

Total number of vaginal swabs obtained for bacterial isolation was 40 at the time of insertion and 40 at the time of implant removal, in total 80 vaginal swabs were obtained. All (100%) the vaginal swabs (Insertion/removal) in all the groups of animals using different protocols yielded growth of bacteria in all the vaginal swabs. Number of bacterial isolates obtained in each group of anestrus cattle at the time of insertion and removal of CIDR, TRIU-B, P<sub>4</sub> Sponge and Placebo Sponge were: 20, 21; 22, 23; 21, 22 and 25, 26 respectively with total sum of 180 isolates. The highest rate of isolation of bacteria

per sample was recorded in the placebo group at the time of removal of placebo (2.60), followed by (2.50) same group and TRIU-B group (insertion), at the time of insertion in CIDR and P<sub>4</sub> Sponge groups (2.2) and lowest in (2) CIDR, TRIU-B and P<sub>4</sub> Sponge groups at the time of removal of implants. In most of the groups the bacterial isolation showed predominance of mixed isolates over single isolates 63 (78.75%) vs 17 (21.25%), indicating that in most of the cow vagina harbours mixed bacteria than single isolates (Fig. 4, Table-1).



**Fig 4:** Bar diagram showing recovery of isolates from vaginal swabs as single or mixed cultures

**Table 1:** Recovery of isolates from vaginal swabs of postpartum anestrus cows in different estrus induction protocols as single or mixed cultures

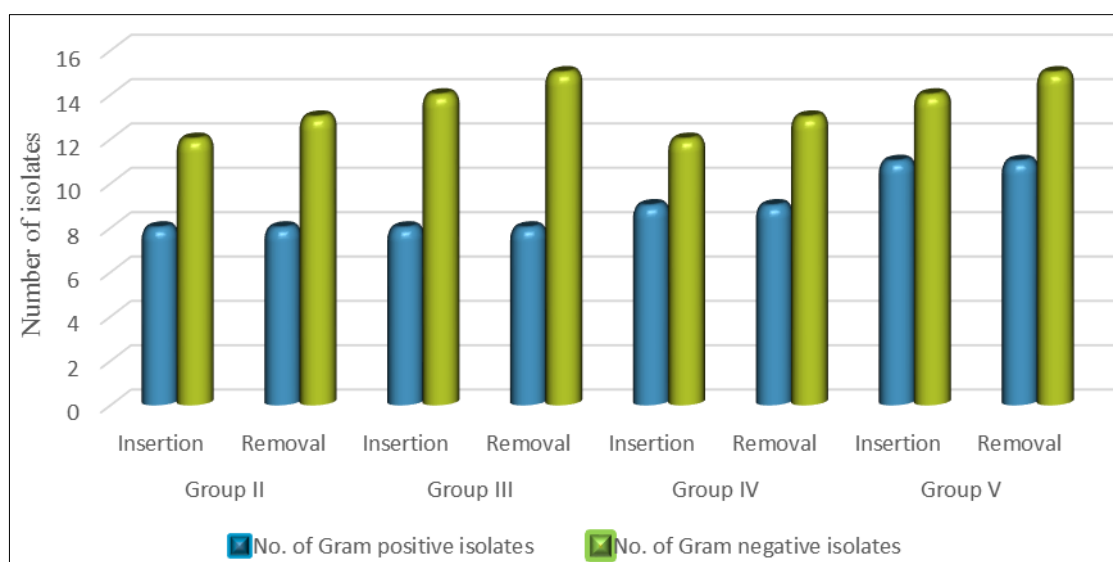
Group	Day	No. of single cultures	No. of mixed cultures	Total no. of swabs
CIDR Protocol	Insertion	2	8	10
	Removal	3	7	10
TRIU-B Protocol	Insertion	1	9	10
	Removal	1	9	10
P <sub>4</sub> Sponge Protocol	Insertion	2	8	10
	Removal	3	7	10
Placebo	Insertion	2	8	10
	Removal	3	7	10
Total	No.	17	63	80
	%	21.25	78.75	100

Out of the 180 bacterial isolates, Gram positive organisms isolated were (72 isolates) 40.00% and Gram-negative

bacteria were (108 isolates) 60.00%, indicating dominance of Gram- negative bacteria (Fig. 5, Table -2).

**Table 2:** Recovery of isolates from vaginal swabs of postpartum anestrus cows in different estrus induction protocol based on Grams reaction.

Group	Day	No. of Gram +ive isolates	No. of Gram -ive isolates	Total no. of isolates
CIDR Protocol	Insertion	8	12	20
	Removal	8	13	21
TRIU-B Protocol	Insertion	8	14	22
	Removal	8	15	23
P <sub>4</sub> Sponge Protocol	Insertion	9	12	21
	Removal	9	13	22
Placebo	Insertion	11	14	25
	Removal	11	15	26
Total	No.	72	108	180
	%	40	60	100



**Fig. 5:** Bar diagram showing recovery of isolates from vaginal swabs based on Grams reaction.

*E. coli*, *Staphylococcus*, *Proteus spp* and *Bacillus spp* were the commonest isolates obtained before and after insertion of CIDR, TRIU-B, P<sub>4</sub> Sponge protocols and placebo sponge in post-partum anestrus cattle. These organisms could, therefore, be considered as a part of the normal vaginal bacterial flora of the cow. The implant used and time/period of isolation did not alter the types of bacteria isolated. From the quantitative study of the organisms from postpartum anestrus cattle before and after insertion of Crestar, CIDR, TRIU-B, P<sub>4</sub> Sponge protocols and placebo sponge it was observed that 8 out of 8 types of bacterial isolates are common before and after insertion of implants. Upon comparing each species of bacteria between before and after insertion of implants it is

observed that there was no remarkable difference in the frequency of occurrence of the different species. This signifies that there is a "normal" bacterial population resident in the vaginal mucus of postpartum anestrus animals. However, *Pseudomonas spp*, was not recovered before and after insertion of TRIU-B implant but it was recovered from vaginal samples before and after insertion of all other vaginal implants including placebo implant in placebo group. Likewise, *Klebsiella*, *Streptococcus* and Coagulase Negative *Staphylococcus* (CNS) was not recovered before and after insertion of Placebo group, CIDR and P<sub>4</sub> Sponge and TRIU-B implants respectively (Table- 3).

**Table 3:** Prevalence of various bacterial genera in vaginal swabs of postpartum anestrus cows during different estrus induction protocols

Protocol	Day	<i>E. coli</i>	<i>Prot.</i>	<i>Pseud.</i>	<i>Kleb.</i>	<i>Staph.</i>	<i>Strept.</i>	<i>Bacil.</i>	<i>CNS</i>	Total no. of isolates
CIDR Protocol	Insertion	7(35.0)	3(15)	1(5)	1(5)	5(25)	0(0.0)	1(5)	2(10)	20
	Removal	7(33.3)	4(19.05)	1(4.7)	1(4.7)	5(23.8)	0(0.0)	1(4.7)	2(9.4)	21
TRIU-B Protocol	Insertion	7(31.8)	5(22.7)	0(0.0)	2(9.1)	6(27.3)	1(4.5)	1(4.5)	0(0.0)	22
	Removal	8(34.8)	5(21.7)	0(0.0)	2(8.7)	6(26.1)	1(4.3)	1(4.3)	0(0.0)	23
P <sub>4</sub> Sponge Protocol	Insertion	6(28.6)	3(14.3)	2(9.4)	1(4.7)	5(23.8)	0(0.0)	2(9.4)	2(9.4)	21
	Removal	7(31.8)	3(13.6)	2(9.1)	1(4.5)	5(22.7)	0(0.0)	2(9.1)	2(9.1)	22
Placebo	Insertion	7(28.0)	5(20.0)	2(8.0)	0(0.0)	7(28.0)	1(4.0)	2(8.0)	1(4.0)	25
	Removal	8(30.7)	5(19.2)	2(7.7)	0(0.0)	7(26.9)	1(3.8)	2(7.7)	1(3.8)	26
Total		57(31.7)	33(18.3)	10(5.0)	8(4.0)	46(25.6)	4(2.0)	12(6.7)	10(5.0)	180

The number in parenthesis indicate percentage of bacterial species obtained from vagina of cow out of total number of isolates obtained at the time of insertion or removal of implant/sponge. Prot = Proteus, Pseud. = Pseudomonas, Kleb. = Klebsiella, Staph. = Staphylococcus, Strept. = Streptococcus, CNS = Coagulase Negative Staphylococcus.



#### 4. Discussion

The extent of Vaginal samples found positive (100%, 40/40) for the presence of bacterial isolates in vaginal swabs in the present study closely corroborated with the observations reported by Kavyashree (2013) [18] and Husted (2003) [16] who reported 100% bacterial culture from the vaginal samples. While these findings are comparatively higher than previous reports varying from 64 to 99 % by Bulman *et al.* (1978) [4], and Dholakia *et al.* (1987) [10]. However, the results obtained in the present study are very higher than those varying from 3.79 to 60 % as reported by Khasatiya *et al.* (1999) [19], Patel *et al.* (2005) [24] and Ocando *et al.* (2010) [20]. The overall ratio of number of isolates per vaginal sample obtained in the present study was 2.25. The highest rate of isolation of bacteria per sample was recorded in the placebo group at the time of removal of placebo (2.60), followed by - (2.50) same group at the time of insertion, (2.3) at the time of TRIU-B removal, (2.2) at the time of TRIU-B insertion and P<sub>4</sub> Sponge removal, (2.1) in CIDR at the time of removal and P<sub>4</sub> Sponge insertion and lowest in (2) CIDR group at the time of insertion of implant. The rate of bacterial isolation per sample reported in the previous studies were 1.30 (Decun and Rosu, 1973) [8], 1.90 (Heist and Tanabe, 1974) [14] and (Panangala *et al.*, 1978) [23] 2.89 while Kavyashree (2013) [18] obtained 2.2 isolates per vaginal samples of normal fertile cows,

The frequency of single and mixed isolates observed in different groups of anestrus animals at the time of insertion and removal of implants in the present study revealed predominance of mixed isolates over single isolates 63 (78.75%) vs 17 (21.25%), indicating dominance of mixed isolates over single isolates (Table.1 and Fig.4). This is in corroboration with the findings of Panangala *et al.* (1978) [23] who also recovered 93.10% and 6.90% of mixed and single bacterial isolates respectively. However, the present findings are contrary to those of Shah and Dholakia (1983) [30], Sharma *et al.* (2008) [31] and Ocando *et al.* (2010) [20] who recorded 34.18%, 11.43% and 25.49% of mixed isolates as compared to 65.82%, 88.57% and 28.43% of single isolates respectively.

Out of the 180 bacterial isolates, Gram positive organisms isolated were (72 isolates) 40.00% and Gram-negative bacteria were (108 isolates) 60.00%, indicating dominance of Gram- negative bacteria. This confirms the findings of Deka *et al.* (1979) [9] who also reported the predominance of gram-negative bacteria isolated from cervico vaginal mucus of cows. Sharma *et al.* (2008) [31], reported 38.71% and 61.29% respectively gram positive and gram-negative organisms. However, several other studies reported the dominance of Gram-positive organisms being isolated more frequently than gram negative bacteria (Shah and Dholakia, 1983; Petit *et al.*, 2009 and Ocando *et al.*, 2010) [30, 25, 20]. However, Ramaswamy *et al.* (1998) [27] and Azawi *et al.* (2008) [3] reported that prevalence of gram positive and Gram-negative organisms was almost equal.

*E. coli*, *Staphylococcus*, *Proteus spp* and *Bacillus spp* were the commonest isolates obtained before and after insertion of CIDR, TRIU-B, P<sub>4</sub> Sponge protocols and Blank sponge in post-partum anestrus cows. These organisms could, therefore, be considered as a part of the normal vaginal bacterial flora of the cow. Kather *et al.* (2012) [17] observed that in multiparous cows *E. coli* was the most prevalent bacteria with an isolation rate (38.3%), followed by *S. aureus* (20.0%) and *Proteus spp.* (10.0%). Other bacteria in order and frequency were *Pseudomonas spp.*, *Klebsiella spp.*, *Bacillus spp.* which is in

close agreement with the present study. Further, some workers (Kather *et al.*, 2012; Ata *et al.*, 2010; Hella 2014) [17, 1, 13] observed that *E. coli* and *Staphylococcus* were the predominant isolates from intact vagina which is in agreement with the present findings. However, present results are in disagreement with other previous studies (Gani *et al.*, 2008) [12], where *Staphylococcus* was predominant 37.8%. Also, the main source of vaginal bacteria is variable according to species including contamination from environment, skin or faecal materials (Torres *et al.*, 1994) [32]. Carmona *et al.* (1993) [5] mentioned that the differences of the microflora between clinically healthy and sick cows, between cows with normal or abnormal deliveries or between cows and heifers were not significant. A few studies have been conducted, however, that evaluated the normal flora in healthy cattle (Amin *et al.*, 1996; Otero *et al.*, 1999, and Otero *et al.*, 2000) [2, 21, 22]. These studies demonstrated that the dominant bacteria were *Streptococcus spp.* followed by coagulase-negative *Staphylococcus spp.* (White *et al.*, 1989 and Otero *et al.*, 2000) [33,22], Enterobacteriaceae (in particular, *Escherichia coli*), members of the genus *Lactobacillus* were consistently present but in much lower numbers (Otero *et al.*, 2000) [22].

#### 4. Conclusion

It could be concluded that the use of intravaginal devices, regardless of their composition, may generate changes in the normal vaginal bacterial flora of the vaginal mucus, the implant used and time/period of isolation did not alter the types of bacteria isolated. These organisms could, therefore, be considered as a part of the normal vaginal bacterial flora of the cow and did not reflect on the subsequent fertility.

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The authors are responsible for all the content.

#### 6. Conflict of interest.

The authors declare that they have no conflict of interest.

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