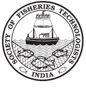

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Pathogenic Vibrios in *Penaeus monodon* Shrimp Hatcheries and Aquaculture Farms

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Abstract

Presumptive *Vibrio* species isolated from hatchery water (105), post-larvae (105), farm water (75), farm sediment (75) and shrimp (100) from *Penaeus monodon* aquaculture systems were screened for the presence of pathogenic *Vibrio* species. *V. alginolyticus* was the most dominant pathogenic *Vibrio* species in both hatchery and aquaculture farm environments. *V. parahaemolyticus*, *V. vulnificus* and *V. harveyi* were detected only in hatcheries and *V. cholerae* was detected only in aquaculture farms. A PCR method was developed to detect common pathogenic vibrios in *P. monodon* aquaculture system utilizing *V. cholerae* species-specific *sodB* primers, *V. cholerae ctxAB* genes specific primers, *V. alginolyticus* specific *gyrB* primers, *V. vulnificus* specific *hsp60* primers and *V. parahaemolyticus* specific *flaE* primers, which can help in rapidly identifying these human pathogenic vibrios from an unknown colony on TCBS agar.

Keywords: *Penaeus monodon*, pathogenic vibrios, *Vibrio alginolyticus*, *V. cholerae*, PCR

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Introduction

Worldwide, penaeid shrimps are considered as having high potential for intensive aquaculture. In India, during the year 2009-10, a total of 95 918.89 t of shrimp was produced from a culture area of 1 02 259.98 ha. Andhra Pradesh (39 537 t) was the leading state in shrimp production (MPEDA, 2010). Frozen shrimp constitutes a significant part of the marine exports. The quantity of frozen shrimp exported from India in 2009-10 was 1 30 553 t which had realized US $ 883.03 million in foreign exchange earnings.

Strict quality guidelines have been laid by the importing nations, for the food products that enter their markets. The mere presence of pathogenic vibrios is sufficient for rejection of the exported product. Bergey's Manual of Systematic Bacteriology (Brenner et al., 2005) lists 44 species under the genus *Vibrio*, of which 12 are pathogenic to humans viz., *V. cholerae, V. vulnificus*, *V. parahaemolyticus*, *V. furnissi*, *V. metschnikovii*, *V. cincinnatiensis*, *V. alginolyticus*, *V. mimicus*, *V. fluvialis*, *V. hollisae*, *V. damsela* and *V. harveyi*. Rapid Alert System for Food and Feed (RASFF) of the European Union has issued alert notifications for the presence of *V. cholerae*, *V. cholerae* Non O1 and Non O139, *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus* and *V. fluvialis* in shrimps imported by the EU countries. During the period 1999 to 2008, a total of 210 alert notifications were issued vis-à-vis shrimp and fish. The presence of *V. parahaemolyticus*, *V. cholerae*, *V. vulnificus* was the sole reason for rejection in 113, 55 and 3 instances, respectively as shown in the RASFF portal of the EU (https://webgate.ec.europa.eu/rasff-window/portal/). However, in many cases, the alert notifications were issued due to the presence of two or more *Vibrio* species in the imported product. RASSF notifications were issued with respect to *Penaeus monodon* and other shrimps exported to EU from India. Pathogenic vibrios have been detected in cultured shrimp and seafood produced in Indian subcontinent (Aravindan & Sheeja 2001; Sanjeev et al., 2000; Jeyasekaran & Ayyappan 2002; Sudha et al., 2002; Rao & Surendran 2010; Smitha et al., 2011). The present study was undertaken to study the

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incidence of human pathogenic vibrios in *P. monodon* hatcheries and aquaculture farms and to develop a PCR based rapid detection method for rapid detection of common pathogenic vibrios in the aquaculture system.

Materials and Methods

Hatchery samples: Water (n=7) and post-larvae (n=7) samples were collected from seven *P. monodon* hatcheries located on the East Coast of India. Post-larvae were brought to the laboratory in live condition in oxygen filled polythene bags.

Aquaculture samples: Farm water (n=5), sediment (n=5) and shrimp (n=5) samples were collected from five *P. monodon* aquaculture farms located on the East Coast of India. Water samples were collected in sterile glass bottles and shrimp were caught by cast net and collected in sterile polythene bags. Sediment samples were collected in sterile bottles. Water, sediment and shrimp samples were brought to the laboratory under iced condition.

Bacteriological analysis: The hatchery and farm samples were analyzed for quantitative and qualitative bacteriological parameters (Kaysner & DePaola, 2004; Brenner et al., 2005). Total plate count (TPC) was determined using tryptone glucose yeast extract agar. Total presumptive vibrio counts (TPVC) was determined by spread plating using appropriate sample dilutions on pre-set surface dried plates of Thiosulfate-Citrate-Bilesalt-Sucrose (TCBS) agar. The yellow and green colonies for each dilution were recorded as presumptive *Vibrio* count. Confirmation of vibrios was done by Gram staining, nitrate test, oxidase test and Hugh and Leifson glucose O/F test. The cultures confirmed as vibrios were identified to the species level initially by using the scheme of Noguerola & Blanch (2008) and confirmed as per the biochemical reactions described in Bergey's Manual of Systematic Bacteriology (Brenner et al., 2005).

Molecular characterization: Duplex-PCR targeting *sodB* and *ctx* genes of *V. cholerae* (Rao & Surendran, 2011), *gyrB* of *V. alginolyticus* (Zhou et al., 2007), *hsp60* of *V. vulnificus* and *flaE* sequence of *V. parahaemolyticus* (Tarr et al., 2007) were also used for molecular confirmation. The *V. cholerae* (MTCC 3906), *V. vulnificus* (MTCC 1145), *V. alginolyticus* (ATCC 17749) and *V. parahaemolyticus* (ATCC 17802) type cultures were used as positive standards for PCR reactions.

PCR for the detection of pathogenic vibrios (Pathogenic vibrio-PCR)

Template prepration: *V. cholerae*, cholera toxin producing *V. cholerae*, *V. vulnificus*, *V. alginolyticus* and *V. parahaemolyticus* were inoculated separately to tubes containing T_1N_1 broth and incubated at 37°C for 24 h. One ml from each T_1N_1 culture was centrifuged at 10 000 rpm for 10 min and the cell pellet was resuspended in 100 µl of Tris-EDTA (TE) buffer. The microcentrifuge tubes were placed in a dry bath at 95°C for 5 min and the crude lysate was used immediately as template for PCR reaction.

Table 1. Oligonucleotide primers used in the detection of pathogenic vibrios

Organism	Primer*	Sequence	Target	Amplicon Size
Vibrio cholerae	F	5′-AAG ACC TCA ACT GGC GGT A – 3′	*sodB*	248 bp
	R	5′-GAA GTG TTA GTG ATC GCC AGA GT – 3′		
Vibrio cholerae	F	5′-TGA AAT AAA GCA GTC AGG TG – 3′	*ctxAB*	777 bp
	R	5′-GGT ATT CTG CAC ACA AAT CAG– 3′		
Vibrio vulnificus	F	5′-GTC TTA AAG CGG TTG CTG – 3′	*hsp*	410 bp
	R	5′-CGC TTC AAG TGC TGG TAG AAG– 3′		
Vibrio parahaemolyticus	F	5′-GCA GCT GAT CAA AAC GTT GAG T – 3′	*flaE*	897 bp
	R	5′-ATT ATC GAT CGT GCC ACT CAC– 3′		
Vibrio alginolyticus	F	5′-ATT GAG AAC CCG ACA GAA GCG AAG– 3′	*gyrB*	340 bp
	R	5′-CCT AAT GCG GTG ATC AGT GTT ACT– 3′		

*All primers were procured from Integrated DNA Technologies (IDT), USA

PCR reaction conditions: A typical 20 μl reaction contained 1.2 μl of crude lysate, 0.5 μM each of *V. cholerae* species specific primers, 0.5 μM each of *ctxAB* primers, 0.25 μM each of *V. vulnificus* primers, 0.16 μM each of *V. alginolyticus* primers and 1 μM each of *V. parahaemolyticus* primers and 18 μl of master reaction mix containing 10 mM Tris-HCl, pH 8.3; 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM each dATP, dCTP, dGTP and dTTP and 1U of *Taq* polymerase. The sequence of the different primers used in pathogenic vibrio-PCR is given in Table 1. The thermal cycling profile was as follows: initial denaturation of template DNA at 93°C for 15 min; 35 amplification cycles wherein each cycle consisted of denaturation at 92°C for 40 sec, primer annealing at 57°C for 1 min and primer extension at 72°C for 1.5 min; final extension at 72°C for 7 min.

Gel electrophoresis: Agarose gel analysis of PCR products was performed as per Sambrook & Russell (2001). PCR product (10 μl) was mixed with 2 μl of 6X gel loading buffer and loaded into sample wells of 2% Agarose gel submerged in 1X TAE containing 1 μg ml^{-1} ethidium bromide. One well was used for loading 100 bp DNA ladder (Bangalore Genei, India). After appropriate migration with constant voltage of 100 volts for 30-45 min, the agarose gel was visualized on a UV-transilluminator.

Results and Discussion

The TPC and TPVC of hatchery water, post-larvae (PL), aquaculture farm water, farm sediment, and shrimp samples are presented in Table 2. Vibrio loads (TPVC) were higher in *P. monodon* hatchery samples than in aquaculture pond samples. Post-larvae had maximum mean loads of vibrios (2.1×10^5 cfu g^{-1}). Shrimp cephalothorax (head) portion had relatively higher mean counts of vibrios (3.5×10^4 cfu g^{-1}) than shrimp muscle portion (1.4×10^4cfu g^{-1}). Hatchery water had higher vibrio loads (2400 cfu ml^{-1}) than farm water (150 cfu ml^{-1}). The vibrio loads in farm water were within the range of previously reported values (Otta et al., 1999; 2001). Liu et al. (1994) observed that in *P. monodon* hatchery, during initial stages, the major bacterial flora were gram positives but after Zoea III stage, the gram negative bacteria become the main flora, of which the vibrio (68%) were dominant species. In aquaculture farms, the mean TPVC of farm sediment (1.5×10^3 cfu g^{-1}) was 10 times higher than the mean TPVC of farm water (1.5×10^2 cfu ml^{-1}) which was similar to previous studies. Sanjeev (1999) reported that in a brackishwater culture pond, *V. parahaemolyticus* count was 460 MPN ml^{-1} in water but in sediment the count was 2.4×10^4 MPN g^{-1}. The number of vibrio in farm sediment was reported to be 10 to 20 times higher than those in water column (Li et al., 2002). The dominant species (47.5%) belonged to the genus *Vibrio* in water samples from *P. monodon* pond in Taiwan (Wei & Hsu, 2001).

In shrimp hatchery samples, the post-larvae (2.2×10^6 cfu g^{-1}) had higher bacterial load than water (5.6×10^3cfu ml^{-1}). The TPC of hatchery waters obtained

Table 2. Total plate counts (TPC) and total presumptive vibrio counts (TPVC) in *Penaeus monodon* hatcheries and aquaculture farms

Source	Total Plate Count	Total presumptive Vibrio Count
Hatchery water, cfu ml^{-1} (n=7)	$5.6 \times 10^3 \pm 3.9 \times 10^3$* ($1.6 \times 10^3$ to 1.2×10^4)**	$2.4 \times 10^3 \pm 2.2 \times 10^3$ * (6.6×10^2 to 2.8×10^3)**
Post Larvae from Hatcheries, cfu g^{-1}; (n=7)	$2.2 \times 10^6 \pm 1.9 \times 10^6$ (9.2×10^5 to $4.5\ 10^6$)	$2.1 \times 10^5 \pm 1.1 \times 10^5$ (7.6×10^4 to 3.7×10^5)
Aquaculture Farm water, cfu ml^{-1}; (n=5)	$3.5 \times 10^3 \pm 7.9 \times 10^2$ (2.6×10^3 to 4.4×10^3)	$1.5 \times 10^2 \pm 42$ (80 to 2×10^2)
Aquaculture Farm Sediment, cfu g^{-1}; (n=5)	$2.9 \times 10^5 \pm 1.4 \times 10^4$ (2.8×10^5 to 3.0×10^5)	$1.5 \times 10^3 \pm 6.4 \times 10^2$ (8×10^2 to 2.2×10^3)
Shrimp Cephalothorax, cfu g^{-1}; (n=5)	$4.78 \times 10^5 \pm 3.0 \times 10^4$ (4.4×10^5 to 5.1×10^5)	$3.5 \times 10^4 \pm 2.2 \times 10^4$ (1.7×10^4 to 7.0×10^4)
Shrimp Muscle, cfu g^{-1}; (n=5)	$2.7 \times 10^5 \pm 2.0 \times 10^4$ (2.4×10^5 to 2.9×10^5)	$1.4 \times 10^4 \pm 1.42 \times 10^4$ (1.8×10^3 to 3.6×10^4)

* mean ± SD

** value in parentheses indicates range

in this study was lower than the values reported by Otta et al. (2001), which might be due to the better water management systems adopted by the shrimp hatcheries. Abraham & Palaniappan (2004) noticed a gradual but significant increase in the mean total viable counts from eggs (log 4.92 cfu g^{-1}) to post-larvae (log 7.00 cfu g^{-1}). In aquaculture farm samples, the mean TPC of farm sediment (2.9 x 10^5 cfu g^{-1}) was 2 logs higher than pond water (3.5 x 10^3 cfu ml^{-1}). Shrimp head had relatively higher bacterial load (4.8 x 10^5 cfu g^{-1}) when compared to shrimp muscle (2.7 x 10^5 cfu g^{-1}). The bacterial counts in sediment were reported as higher than those in water column (Dalmin et al., 2002; Li et al., 2002). The higher bacterial loads in pond sediments obtained in this study could be attributed to the accumulation of organic matter at the pond bottom which stimulated bacterial growth.

V. alginolyticus was the most common species detected in hatchery samples followed by *V. vulnificus*, *V. parahaemolyticus* and *V. harveyi* (Table 3). *V. alginolyticus* was the most dominant pathogenic *Vibrio* species in hatchery water and post-larvae samples. *V. harveyi* was detected in hatchery waters (3.85%) but not in post-larvae. The luminous vibrios *V. harveyi*, *V. fischeri* and *V. splendidus* are important shrimp pathogens. The primary source of *V. harveyi* in a shrimp hatchery was the faecal matter from brood stock, possibly at the time of spawning (Abraham & Palaniappan, 2004) and artemia (Vaseeharan & Ramasamy, 2003). *V. alginolyticus* and *V. harveyi* were isolated from shrimp-farm water, sediment, shrimp larvae and hatchery water samples (Kumar et al., 2007). The lower incidence of *V. harveyi* in hatchery samples in the present study can be attributed to better hatchery water quality management and the size of the post-larvae samples used for analysis. The post-larvae size was above PL-15 and healthy and had attained the marketable size for stocking in shrimp culture farms.

V. alginolyticus (38.4%) was the most common pathogenic vibrio species detected in aquaculture samples followed by *V. cholerae* (16.8%) (Table 3). Other pathogenic vibrio species that were detected in hatchery samples *viz., V. parahaemolyticus, V. vulnificus* and *V. harveyi* were not detected in aquaculture farms. *V. alginolyticus* was the most predominant pathogenic vibrio species in farm sediment, farm water and shrimp (whole) samples (Table 3). *V. cholerae* incidence was higher in farm water (7.6%) than in farm sediment (5.2%) and shrimp (whole) (4%). Shrimp head portion had relatively higher incidence of *V. alginolyticus* (6%) and *V. cholerae* (3.6%) when compared to shrimp muscle where the incidence levels were 2.4 and 1.6% respectively.

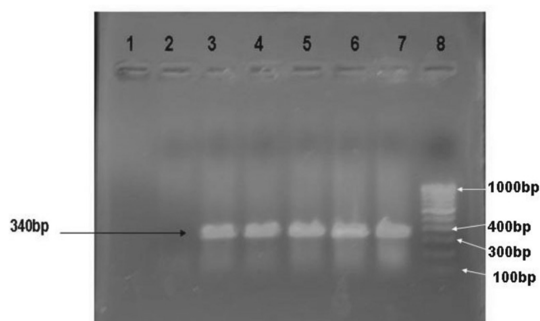
The incidence of total pathogenic vibrios in aquaculture samples was higher than hatchery samples but the diversity of vibrios was higher in hatchery environment than in aquaculture farms. Species-wise, *V. alginolyticus* was the most commonly encountered pathogenic vibrio species in hatchery (24.3%) and farm (38.4%) samples. *V. parahaemolyticus*, *V. vulnificus* and *V. harveyi* were found only in

Table 3. The incidence of pathogenic vibrio species in *Penaeus monodon* hatchery and aquaculture samples

Pathogenic *Vibrio* spp.	*Penaeus monodon* hatchery samples		Aquaculture farm samples		
	Hatchery water* (%)	Post-larvae* (%)	Pond water** (%)	Pond sediment** (%)	Shrimp (whole)** (%)
Vibrio alginolyticus	17	7.1	12.4	16.4	9.6
Vibrio vulnificus	6.7	2.4	0	0	0
Vibrio parahaemolyticus	7.1	1.4	0	0	0
Vibrio harveyi	3.8	0	0	0	0
Vibrio cholerae	0	0	7.6	5.2	4.0
Other non-pathogenic group of *Vibrio* spp.	15.2	39.1	10	8.4	26.4

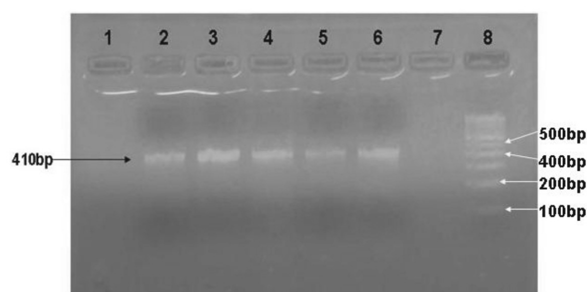
* Values as percentage of the total vibrios isolated from shrimp hatcheries (n=210 isolates)
** Values as percentage of the total vibrios isolated from aquaculture farms (n=250 isolates)

hatchery samples and while they were totally absent in the farm samples. Similarly, *V. cholerae* was not detected in hatchery samples but it was found in farm samples. However, all the *V. cholerae* belonged to the non O1/non-O139 serogroup (Rao & Surendran, 2011). The pathogenic vibrio species isolated in this study from shrimp farms were similar to previously reported results. Bhaskar et al. (1998) noticed that *V. alginolyticus* was the most common vibrio species (57% incidence) in the farming phase of tiger shrimp (*P. monodon*). *V. cholerae, V. parahaemolyticus* and *V. vulnificus* were the other species encountered. *V. alginolyticus* (28.8%) was the dominant flora among the vibrio isolates (n=278) obtained from shrimp farming systems of Tamil Nadu (Felix, 2000). Jayasree et al. (2006) surveyed *P. monodon* culture ponds of coastal Andhra Pradesh and isolated six species of vibrios viz., *V. harveyi, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus,* and *V. splendidus*. Farm-made feeds showed a high incidence of *V. parahaemolyticus*, *V. cholerae*, *E. coli* and *Staphylococcus aureus* (Raghavan, 2003). Five species of vibrio viz., *V. alginolyticus, V. parahaemolyticus, V. vulnificus, V. fluvialis* and *V. mimicus* were detected in the pond water and the prawn body with *V. alginolyticus* and *V. parahaemolyticus* as the dominant species for all ponds (Ni et al., 1995). Dalsgaard et al. (1995) isolated 143 *V. cholerae* non O1 strains from shrimp farms in Thailand.



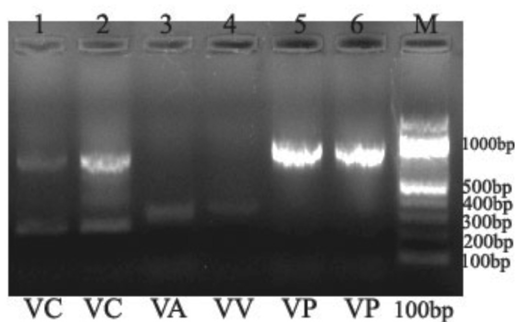
a) PCR for detection of *Vibrio alginolyticus*

Lane 2 : Negative control,
Lane 3 to 6 : *Vibrio alginolyticus* isolates from aquaculture farms (340 bp),
Lane 7 : *Vibrio alginolyticus* (ATCC 17749)
Lane 8 : 100 bp DNA ladder



b) PCR for detection of *Vibrio vulnificus*

Lane 2 to 5 : *Vibrio vulnificus* isolates from shrimp hatcheries (410 bp),
Lane 6 : *Vibrio vulnificus* (MTCC 1145),
Lane 8 : 100 bp DNA ladder



c) Multiplex PCR for the detection of pathogenic vibrios

Lane 1 & 2 : Cholera toxin producing *Vibrio cholerae* (248 bp & 777 bp);
lane 3 : *Vibrio alginolyticus* (340 bp);
lane 4 : *Vibrio vulnificus* (410 bp);
lane 5 : *Vibrio parahaemolyticus* (897 bp);
lane 6 : *Vibrio parahaemolyticus* (897 bp);
lane 8 : 100 bp DNA ladder

Fig 1. PCR for the detection of pathogenic vibrios

The interaction of virus and *V. alginolyticus* in the earlier stage of virus disease of *P. chinensis* showed that insidious infection of vibrio is advantageous to the infection of virus (Ding et al., 2000). White shrimp *Litopenaeus vannamei* transferred from 25 ppt to low salinity levels (5 and 15 ppt) had reduced immune ability and decreased resistance against *V. alginolyticus* infection (Wang & Chen, 2005). The farming of *L. vannamei* is rapidly gaining momentum in India, especially in Andhra Pradesh. The role of *V. alginolyticus* as a predisposing factor for viral disease assumes greater significance as it is the most predominant vibrio in the shrimp culture system.

A PCR method was developed to detect common pathogenic vibrios. Species-specific house keeping genes were the target sites for amplification. Standard PCR using one set of specific primers was performed to detect *V. alginolyticus* (Fig 1a), *V. vulnificus* (Fig 1b), *V. parahaemolyticus* and *V. cholerae* (Fig. 1c).

Pathogenic vibrio-PCR was performed with a PCR master mix containing all the 5 sets of primers. The primer concentrations of 0.5 μM each of *V. cholerae* species specific primers, 0.5 μM each of *ctxAB* primers, 0.25 μM each of *V. vulnificus* primers, 0.16 μM each of *V. alginolyticus* primers and 1 μM each of *V. parahaemolyticus* primers was found sufficient for obtaining detectable result by pathogenic vibrio-PCR. The annealing temperature for *ctxAB* primers was 55°C (Koch et al., 2001), the annealing temperature for *sod*B, *hsp*60 *and fla*E primers was 57°C (Tarr et al., 2007) and the annealing temperature for gyrB primers was 60°C (Zhou et al., 2007). As the annealing temperatures of the three different PCR methods were closer, the pathogenic vibrio-PCR was planned to include all these primers in a single reaction with an annealing temperature of 57°C.

When specific DNA was added as template to the PCR mix, only the corresponding primers specifically reacted and yielded that particular amplicon *viz.*, single amplicon in the presence of multiple primers. Non cholera toxin producing *V. cholera* cultures yielded an amplicon of 248 bp; *ctxAB* positive *V. cholerae* cultures yielded two amplicons *viz.*, 248 bp and 777 bp; *V. alginolyticus* cultures yielded an amplicon of 340 bp; *V. vulnificus* cultures yielded an amplicon of 410 bp and *V. parahaemolyticus* cultures yielded an amplicon of 897 bp (Fig. 1c). Pathogenic vibrio-PCR can help in rapidly identifying the above mentioned human pathogenic vibrios from an unknown colony on TCBS agar. Normal time for detection employing biochemical tests range between 3 and 4 days whereas PCR analysis can be completed within a day.

In this study, it was observed that *V. alginolyticus* was the most dominant pathogenic *Vibrio* species both in *P. monodon* hatcheries and aquaculture farms. *V. parahaemolyticus*, *V. vulnificus* and *V. harveyi* were detected only in *P. monodon* hatcheries and *V. cholerae* was detected only in *P. monodon* aquaculture farms. Non O1/non-O139 *V. cholerae* seems to be the major potential risk that can be expected in black tiger raw shrimps.

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