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Histopathology and molecular detection of *Brucella melitensis* Infection in small ruminants

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ABSTRACT

The present study was planned to detect *Brucella melitensis* (*B. melitensis*) infection in small ruminants and related histopathological lesions in organs. Twenty three seropositive sheep and goats which were, obligatory slaughtered at different abattoirs in El- Menofia governorate, Egypt were used. Confirmed diagnosis of *B. melitensis* was carried out using bacterial isolation and biochemical identification with application of AMOS PCR. The study revealed that 5 animals; 5/23 (22 %) were confirmed positive for *B. melitensis*. Histopathologically, granulomatous and pyogranulomatous reactions, interstitial fibrosis, multifocal necrosis with occasional dystrophic calcification were characteristic lesions of the examined endometrium, mammary glands and lymph nodes. The testis of infected ram revealed testicular degeneration with mild interstitial lymphocytic and histiocytic infiltration. The lymph nodes showed marked lymphoid depletion and diffuse macrophages infiltrates of the medullary sinuses. The study concluded that isolation and identification of organisms with PCR confirmation are the diagnosis gold standard and *B. melitensis* infection in sheep and goats characterized microscopically by chronic inflammatory reaction in addition to widespread necrosis in different organs.

1. INTRODUCTION

Brucellosis is a worldwide distributed zoonotic bacterial disease affecting a wide range of mammals including humans (Cutler et al. 2005). *Brucella melitensis* (*B. melitensis*) is considered the most virulent species causing severe disease in humans, small ruminants and bovines, while cross-species transmission has been proved (Hashemifar et al. 2017; De Massis, et al. 2019). Although continuous progress in control of brucellosis is notable, it is still of great economic importance (Rossetti, et al. 2017; Ebid, et al. 2020). Because of this alarming situation, the world organizations consider brucellosis a significant public health problem (Wareth, et al. 2019). The disease has been endemic in Egypt for many years. Thus, the use of serological test is recommended as a mean to obtain indirect proof of the infection. However, standardized conditions suitable for the diagnosis of cattle infection are not adequate in sheep and goats (Yahaya et al. 2019). Accordingly, the present study focused specifically on samples obtained from not only slaughtered seropositive sheep and goats, but also after isolation of *B. melitensis* and detection of their antigens. *B. melitensis* causes abortion in female goats and sheep with unilateral orchitis in case of males (Alton, 2015). Grossly, granulomatous inflammatory lesions were present in the reproductive organs (Saxena et al., 2018). However, there are a lack of studies on the pathology of natural brucellosis in sheep and goats, especially, those aim to assess a wide range of detailed histopathological lesions in infected organs.

Therefore, the present study was planned to identify *B. melitensis* in seropositive sheep and goats slaughtered at different abattoirs in El- Menofia governorate through isolation and biochemical identification, detection of *B. melitensis* in the isolated strains by PCR examination and to describe the histopathological lesions in different organs of infected animals.

2. MATERIAL AND METHODS

2.1. Samples collection

The present study was carried out on 23 samples [17 sheep (14 females and 3 males) and 6 goats (females) were examined during the period from June 2018 to December 2019]. The samples were obtained from *Brucella* seropositive sheep and goats, obligatory slaughtered at different abattoirs in El- Menofia governorate. These animals were previously tested using the Rose Bengal Plate Test (RBPT), Buffered Acidified Plate Antigen Test (BAPAT); followed by complement fixation test (CFT) and ELISA as confirmatory tests according to discarding culling system of the Egyptian Veterinary Services. In the present study analyzed 23 animals from seropositive sheep and goats; included Lymph nodes of (uterine, supra-mammary, testicular and retropharyngeal) were tested for *Brucella* using bacteriological culture and confirmed with PCR technique.

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Table (1): The prevalence of S. *aureus* strains in the collected samples

Species	Location	Sex	Age (Years)	Organs	Total samples
Sheep	Birket El-Saba	Female	4	- Uterus - Mammary gland - Uterine lymph node - Supra mammary lymph nodes - Retropharyngeal lymph node	5
Sheep	Birket El-Saba	Female	4	- Uterus - Mammary gland - Uterine lymph node - Supra mammary lymph nodes - Retropharyngeal lymph node	5
Goat	Birket El-Saba	Female	2	- Uterus - Mammary gland - Uterine lymph node - Supra mammary lymph nodes - Retropharyngeal lymph node	5
Goat	Menouf	Female	2	- Uterus - Mammary gland - Uterine lymph node - Supra mammary lymph nodes - Retropharyngeal lymph node	5
Sheep	Shebin el kom	Male	3	- Testis - Epididymis - Testicular lymph node - Retropharyngeal lymph node	4

Table (2): Specific oligonucleotide primer sequence used in this study

Gene	Target agent	Sequence	Amplified product (bp)	Reference
IS711	*B. abortus*	1S711-specificPrimer TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT B. abortus-specific Primer GAC-GAA-CGG-AAT-TTT-TCC-AAT-CCC	498	**Bricker and Halling, 1994**
	B. melitensis	1S711-specificPrimer TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT B. melitensis-specific Primer AAA-TCG-CGT-CCT-TGC-TGG-TCT-GA	731	
	B. ovis	1S711-specificPrimer TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT B. ovis-specific Primer CGG-GTT-CTG-GCA-CCA-TCG-TCG	976	
	B. suis	1S711-specificPrimer TGC-CGA-TCA-CTT-AAG-GGC-CTT-CAT B. suis-specific Primer GCG-CGG-TTT-TCT-GAA-GGT-TCA-GG	285	

Samples from confirmed infected animals, included 4 uteri, 4 mammary glands, 4 uterine lymph nodes, 4 supra-mammary lymph nodes (2 sheep and 2 goat), 5 retropharyngeal lymph nodes (3 sheep and 2 goat), one testis, one epididymis, and one testicular lymph node (male sheep) were taken for pathological investigation. All data about the samples as animal species, age, sex and locality of collection were recorded in Table (1). The samples were transferred to the lab in ice bags as quick as possible for bacteriological and PCR examination. Furthermore, tissue specimens from all collected organs were fixed in neutral buffered formalin for histopathological examination.

2.2. Bacteriological examination

2.2.1. Isolation of Brucella spp.

Briefly, isolation was performed by direct culturing of lymph nodes on selective *Brucella* agar and incubation at 37 °C with 5 % CO_2. Media were routinely examined on the 4th day and upwards every 48 hours before being discarded as negative after 3 weeks; the suspected colonies were further identified and sub-cultured on *Brucella* agar (Alton et al., 1988).

2.2.2. Biochemical identification

The isolates were typed according to CO_2 requirement, H_2S production, Oxidase, Catalase, Urease tests and Gram reaction (Alton et al., 1988).

2.3. Application of AMOS PCR

DNA was extracted with the high pure PCR template preparation kit (Roche Applied Sciences, Mannheim, Germany) according to the manufacture instructions. The AMOS PCR (*B. abortus*, *B. melitensis*, *B.ovis* and *B. suis* PCR) was performed as described before (Bricker and Halling, 1994). The oligonucleotide primers used in this study are shown in table (2).

2.4. Histopathology

Small tissue specimens were collected from (Uterus, Mammary gland, Testis, Epididymis, lymph nodes) were fixed and were processed (washed, dehydrated in ascending grades of ethyl alcohol, cleared in Xylene), followed by embedding in paraffin wax, sectioned at 4 µm and stained with Harris' haematoxylin and eosin (HE). Masson's trichrome staining technique was used to confirm the

presence of collagen fibers following Bancroft and Gamble, (2008).

3. RESULTS

3.1. Bacteriological examination

The result of bacteriological examination revealed that *Brucella* was isolated from 5 animals (3 sheep and 2 goats) out of 23 seropositive sheep and goats (22 %).

The suspected colonies on *Brucella* selective media were round, 1-2mm. in diameter, with smooth margins, round edges, translucent convex, when viewed from above and of golden colour (pale honey-colored).

Microscopical examination of isolates revealed Gram-negative cocobacilli. Biochemically, all isolates exhibited positive results with catalase (air bubbles on plate), oxidase (blake or dark color of Oxidase paper), Urease (rose color after 5-30 min), and H_2S test (black color of H2S paper).

3.2. PCR examination

AMOS-PCR confirmed the detected *Brucella melitensis* in isolates of 5/23 (22 %) seropositive sheep and goats. DNA of *Brucella melitensis* was detected at 731 bp. (Fig. 1) of bacterial culture isolated from lymph nodes of five sheep and goats (2 female sheep, 2 female goats. 1 male sheep)

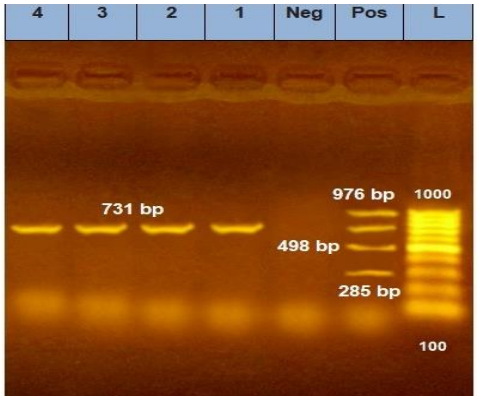

Fig. 1: PCR detection of *Brucella melitensis* from lymph nodes culture of sheep and goats. Lane M: Molecular weight marker (100 bp to 1000bp); Lane Neg: Negative control; Lane Pos: positive control of *B. Abortus* at 498 bp, *B. melitensis* at 731 bp *B. ovis* at 976 bp, *B. suis* at 285 bp, and Lanes 1-2 (female sheep) 3-4 (female goat): Samples tested positive for *B. melitensis* at 731 bp.

3.3. Histopathological findings

Uterus: Multi-focally, within the endometrium and submucosa there were sharply demarcated areas of granulomatous reaction, formed from central caseous necrosis surrounded by numerous epithelioid cells, lymphocytes, few plasma cells and surrounded by abundant fibrous connective tissue (Fig. 2A). Occasionally, caseated center of the granuloma was replaced by degenerate and viable neutrophils aggregates, and surrounded by a zone of same mononuclear inflammatory cells (Fig. 2B). Marked infiltration with macrophages, lymphocytes, plasma cells were observed in goats, in perivascular and peri-glandular connective tissue. Multifocally, the endometrial stroma was expanded by fibrous connective tissue (Fig. 2C). There was glandular depletion; while the survived glands were atrophic or cystic because of peri-glandular fibrosis. Rarely, there was exfoliation of glandular epithelium in lumen mixed with mononuclear inflammatory cells. Moreover, vascular lesions characterized by proliferation of the endothelial cells and hyalinization of tunica media were prominent in endometrial and myometrial blood vessels (Fig. 2D).

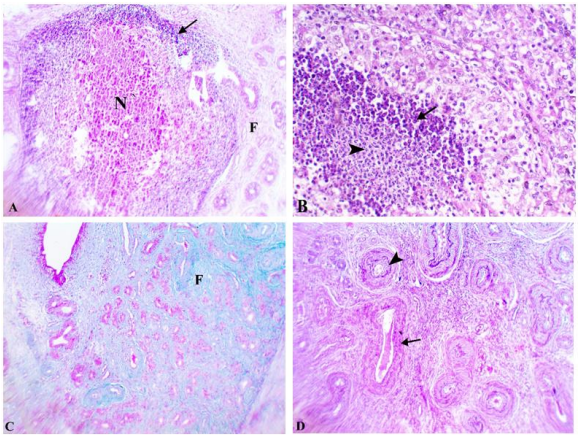

Fig 2:. Uterus of sheep (A, B) and goat (C, D) naturally infected with *Brucella melitensis*. (A) Granuloma with central caseous necrosis (N) surrounded by numerous mononuclear inflammatory cells (arrow) and rimmed by fibrous connective tissue (F), H&E x 200. (B) Aggregates of degenerate (arrowhead) and viable (arrow) neutrophils in the center of pyogranuloma, H&E x 400. (C) Endometrium with increased interstitial fibrosis (F), Masson's trichrome x 100. (D) Endometrial blood vessels with proliferation of endothelial cells (arrowhead) and hyalinization of tunica media (arrow), H&E x 200.

Mammary gland: The mammary gland of sheep revealed multifocal to coalescing areas of necrosis, admixed with dystrophic calcification, affecting and replacing the secretory units (Fig. 3A). Multifocally surrounding the necrotic foci are bands of fibrous connective tissue (fibrosis), moderate numbers of lymphocytes and macrophages and rare plasma cells. There was vacuolar degeneration of epithelial cells lining ducts and adjacent glands characterized by swollen pale vacuolated cytoplasm. Multifocally, glandular lumina and intralobular ducts contained eosinophilic secretion admixed with small amounts of necrotic cellular debris or occasional revealed corpora amylacea characterized by basophilic, concentrically-lamellated foci (Fig. 3B). The lesions in goats were manifested by chronic mastitis (Fig. 3C); with marked infiltration of acini and interstitium with lymphocytes, macrophages and plasma cells. The interlobular septa were thickened and expanded by fibrous tissue proliferation. Moreover, focal areas of caseous necrosis with central dystrophic calcification were also observed (Fig. 3D).

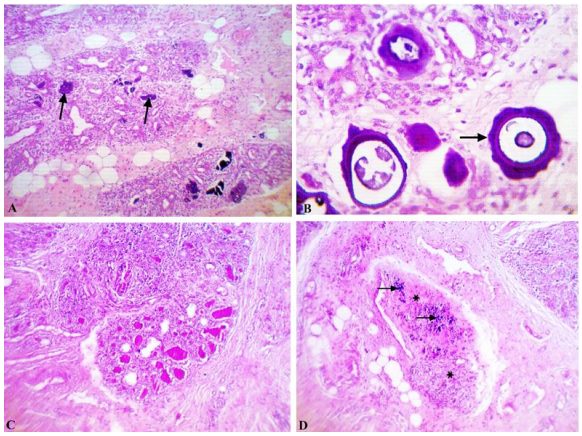

Fig. 3. Mammary gland of sheep (A, B) and goat (C, D) naturally infected with *Brucella melitensis*. (A) Multifocal areas of necrosis and dystrophic calcification (arrow), replacing the secretory units, H&E x 100. (B) Corpora amylacea characterized by basophilic, concentrically-lamellated foci (arrow), H&E x 400. (C) Chronic mastitis, H&E x 100. (D) Focal area of caseous necrosis (asterisk) with central dystrophic calcification (arrow), H&E x 100.

Testis and Epididymis: The testis of infected ram revealed diffuse atrophy of the seminiferous tubules. Multifocally, there were testicular degeneration of small clusters of seminiferous tubules; that was small, hypocellular and reduced spermatogenesis (Fig. 4A). The basement membranes of degenerated seminiferous tubules were thickened and undulating (Fig. 4B), with paucity and vacuolation of germinal cells, and loss of Sertoli cells (Fig. 4C). Occasionally, the lumens of degenerated seminiferous tubules exhibited eosinophilic necrotic debris. The testicular interstitium was expanded by moderate edema mixed with small numbers of lymphocytes and histiocytes. The epididymis showed atrophic tubules, mild interstitial inflammatory cell infiltrates, and there were no spermatids in some epididymal tubules (Fig. 4D).

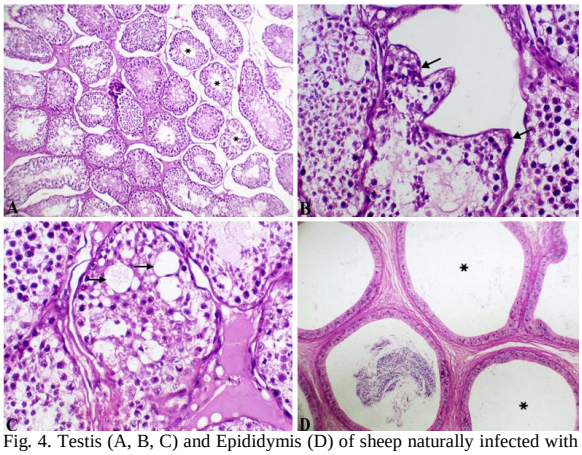

Fig. 4. Testis (A, B, C) and Epididymis (D) of sheep naturally infected with *Brucella melitensis*. (A) Testicular degeneration of small clusters of seminiferous tubules (asterisk) with reduced spermatogenesis, H&E x 100. (B) Thickened and undulating basement membrane (arrow) of degenerated seminiferous tubules, H&E x 400. (C) Vacuolation of germinal cells (arrow) and loss of Sertoli cells, with interstitial oedema (arrow) H&E x 400. (D) Absence of spermatids in epididymal tubules (asterisk), H&E x 200.

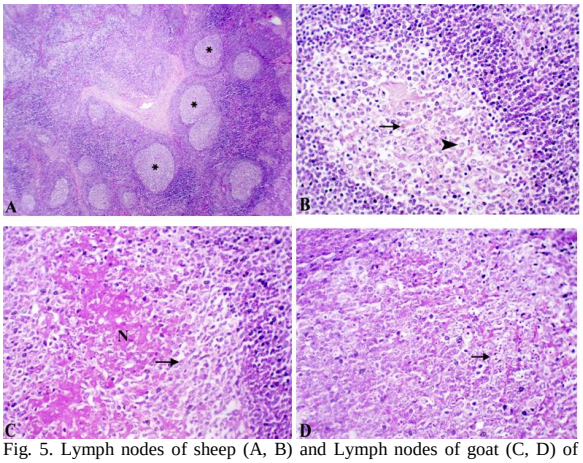

Fig. 5. Lymph nodes of sheep (A, B) and Lymph nodes of goat (C, D) of ram naturally infected with *Brucella melitensis*. (A) Multifocal marked lymphoid depletion (asterisk) of the cortical follicles, H&E x 100. (B) Lymphoid depletion with loss of lymphocytes replaced by eosinophilic cellular and karyorrhectic debris (arrow), fibrin, edema, and numerous macrophages (arrowhead), H&E x 400. (C) Central necrosis (N) of lymphoid elements rimmed by epithelioid macrophages (arrow), H&E x 400. (D) Lymphoid follicles replaced by many tangible body macrophages (arrow) contain apoptotic lymphocytes and cellular debris, H&E x 400.

Lymph nodes: Multifocally, lymph nodes of sheep revealed marked lymphoid depletion within germinal centers of the cortical follicles and para-cortex (Fig. 5A); characterized by loss of lymphocytes (lymphocytolysis) with abundant eosinophilic cellular and karyorrhectic debris, fibrin, edema, and numerous macrophages (Fig. 5B). Diffusely, the medullary sinuses were expanded by increased numbers of macrophages, lymphocytes and plasma cells. Similar changes were also observed in goats, moreover, the center of lymphoid follicles revealed marked focal necrosis of lymphoid elements and rimmed by epithelioid macrophages cells (Fig. 5C), or occasionally replaced by many tingible body macrophages that contained karyolytic lymphocytes with phagocytized eosinophilic cellular and karyorrhectic debris (Fig. 5D)

4. DISCUSSION

B. melitensis is the causative agent of brucellosis in small ruminants and is of considerable economic and public health importance in many countries worldwide. The present study was conducted on *Brucella* seropositive sheep and goats, obligatory slaughtered at different abattoirs in El- Menofia governorate. According to the Egyptian Veterinary Services, RBPT and BAPAT are the current screening tests used for diagnosis of brucellosis in all animals. These serological tests are routinely applied, although they are not completely specific. In this regard, it has to be emphasized that recent study detected *Brucella* DNA in samples of seronegative animals (false-negatives) (El-Diasty, et al. 2018). In the same context, false positive reaction of *B. melitensis* was as a results of cross reaction to other bacteria (Chenais, et al. 2012); or due to the low sensitivity of RBPT antigens in small ruminants (Yahaya, et al. 2019). This observation was realized in the present study where only 5/23 (22 %) of seropositive sheep and goats, confirmed positive by biochemical analysis and PCR technique. Hence, isolation and identification of organisms are the diagnosis gold standard, but it takes long time, and poses a high risk. Therefore, control and eradication of brucellosis from small ruminants in endemic countries require an appropriate serological method for brucellosis diagnosis with PCR confirmation. The histopathological findings in the present study, revealed granulomatous endometritis, formed from central caseation surrounded by numerous epithelioid cells, lymphocytes, few plasma cells and rimmed by fibrous connective tissue; with occasional pyogranulomatous reaction characterized by central aggregates of degenerate and viable neutrophils. These results were in accordance with Abd El-Razik, et al. (2007) who observed nearly the same picture in goats and with Ahmed, et al., (2012) who recorded the same pathological findings in Buffalo-Cows. Previous study suggested that T4SS protein encoded by the VirB operon is essential for induction of microgranuloma formation (Rolán et al. 2009). The pathogenesis of brucellosis proved that the mechanism of injury characterized by cell lysis evolved by pyogranulomatous inflammatory mediators and degradative enzymes. Briefly, infected macrophages transmit the bacteria to trophoblasts and epithelial cells of reproductive tissues. Soon after internalization, *Brucella* spp. are able to survive in phagocytic or non-phagocytic cells and resist the bactericidal mechanisms, by inhibiting the phagosome-lysosome fusion. The successful host cell invasion and intracellular survival or replication of *Brucella* could be attributed to several virulence factors, including urease, type IV secretion system (T4SS), two component regulator system (BvrR/BvrS), cyclic β-1,2-glucans, and LPS (Xavier, et al. 2010). Bacterial growth and replication with concurrent lysis of bacteria-infected macrophages results in pyogranulomatous inflammation of these tissues (Zachary, 2016). The histopathological examination of udder of sheep in this study revealed multifocal to coalescing areas of necrosis and dystrophic calcification, surrounded by fibrosis and mononuclear inflammatory cells; there was

degeneration of epithelial cells lining ducts and secretory glands. The lesions in goats were manifested by chronic interstitial mastitis. According to the available literature there were limited studies about the histopathological impact of chronic brucellosis on the mammary gland of sheep and goats; while acute lesions were described as interstitial mastitis (Shahzad et al., 2018; Jansen, et al. 2020). The fact that mammary gland is a predilection site for brucellosis based on many factors, including the availability of erythritol in high concentrations in infected mammary gland with shedding *B. melitensis* in the milk, that consider of public health risk (Higgins, et al. 2017; Jansen, et al. 2020). The testis of infected ram in the present study revealed atrophy and testicular degeneration of small clusters of seminiferous tubules; with mild interstitial lymphocytic and histiocytic infiltrates in testis and epididymis. Natural cases of necrotizing orchitis and epididymitis caused by *B. melitensis* has been reported in ram (Büyükcangaz, et al. 2013; Saxena, et al. 2018). Acute experimental infection by *B. melitensis* in bucks revealed nearly similar lesions to that observed in this study; additionally, positive immunohistochemistry staining detected *B. meitensis* antigen within the cytoplasm of spermatogonia, Sertoli cells, the surrounding neutrophils and macrophages and necrotic debris (Nasruddin, et al. 2014). The lymph nodes of small ruminants in present study revealed marked lymphoid depletion, lymphocytolysis with central necrosis of lymphoid follicles and diffuse medullary infiltration of macrophages. These findings were coincided with previous studies (Abdel-Razek et al., 2006). Immunohistochemical studies revealed *Brucella* antigen within the cytoplasm of macrophages in lymph nodes (Manrique-Ayala et al., 2021). Studies linked to *Brucella* pathogenicity have been shown that macrophages constitute an important cellular targets of *Brucella*. Activated macrophages are able to kill the internalized *Brucella* after phagocytosis. However, virulent strains as *B. melitensis* are able to replicate within the phagocytic cells (Von Bargen, et al. 2012). Additionally, smooth virulent *Brucella* strain prevented apoptosis of infected macrophages (He et al., 2006), while rough *Brucella* strains induced necrotic cell death and apoptosis for infected macrophages via mitochondrial increased permeability and the release of cytochrome c to cytoplasm (Chen & He, 2009).

5. CONCLUSION

The present study concluded that serological tests routinely applied for diagnosis of brucellosis in sheep and goats are not completely specific. Hence, isolation and identification of organisms with PCR confirmation are the diagnosis gold standard. On the other axis, the histopathological result indicated that natural *B. melitensis* infection in sheep and goats characterized by chronic inflammatory reaction and widespread necrosis in different organs. Interestingly, the detection of numerous macrophages, at different stages of activation infiltrating the tissues sheds light on the important role of these phagocytic cells in pathogenesis of brucellosis.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest for current data

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