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VIRULENCE GENE PROFILING AND PATHOGENICITY OF *Streptococcus agalactiae* ISOLATED FROM TILAPIA, *Oreochromis niloticus* FARMS IN INDONESIA

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ABSTRACT

Streptococcosis caused by *Streptococcus agalactiae* has become a major disease problem in tilapia culture in Indonesia. This study aimed to detect virulence genes of *S. agalactiae* during streptococcosis disease outbreaks in several tilapia farms in Indonesia and evaluate the correlation between biotype and virulence genes to bacterial virulence. The presence of virulence genes was determined in 10 strains of *S. agalactiae* isolated from farm-raised tilapia. Polymerase chain reaction (PCR) protocol was used to determine genes for *cylE*, *hylB*, *lmb*, *bib A*, *PI-2b*, *fbs A*, *fbs B*, *gap*, *PI-1*, and *cfb* in the template DNA. Pathogenicity test was carried out by intraperitoneal injection of 24 hour-cultured *S. agalactiae* to tilapia with 10^8 CFU/fish. Four isolates have seven of virulence genes (*cylE*, *hylB*, *bibA*, *PI-2b*, *fbs A*, *fbs B*, and *gap* genes), three isolates have six virulence genes (*hylB*, *bib A*, *fbs A*, *fbs B*, *gap*, *cfb* genes), one isolate has four virulence gene (*hyl B*, *bib A*, *fbs*, and *cfb* genes), and one isolate has one virulence gene (*PI-2b* gene). None of the isolates has *lmb* or *PI-1* genes. Bacteria with more virulence genes showed higher pathogenicity post injection. Mortality of tilapia injected with β-hemolytic bacteria was 100% within the period of 14-19 hours, while non-hemolytic bacteria was 53.3%-86.6% on 14 days post-injection. Pathological changes associated with the infection by either isolate included melanosis, slow response, anorexia, ocular opacity, gasping, erratic, C-shape, and whirling. It can be concluded that *S. agalactiae* with more virulence genes show a higher level of pathogenicity. The presence of a virulent gene has the potential to be used as a basis for selecting candidate isolates and designing vaccine compositions as an effort to prevent streptococcosis infection in tilapia in Indonesia.

KEYWORDS: ***Oreochromis niloticus*; pathogenicity; *Streptococcus agalactiae*; virulence genes**

INTRODUCTION

Streptococcosis caused by *Streptococcus agalactiae* has been associated with severe economic losses in tilapia culture in Indonesia. Several *S. agalactiae* strains have been isolated from centers of tilapia culture in Indonesia, some showing β-hemolytic ability on blood agar media and others, which are non-hemolytic (Hardi *et al.*, 2011). Recently Suhermanto *et al.* (2019) reported that out of 10 *S. agalactiae* isolates from Indonesia, 40% could be classified as biotype 1 (β-hemolytic) and 60% were biotype-2 (non-hemolytic).

Sheehan *et al.* (2009) classified *S. agalactiae* strains in two biotypes. Biotype-1 which are typically β-hemolytic and biotype-2, which are non-hemolytic. In addition, biotype-1 grewwell at 37°C and hydrolyzed more types of sugars compared to biotype-2. Biotype-2 bacteria were more virulent compared to biotype-1 seen most commonly associated with disease outbreaks. Besides, the geographic distribution of biotype-2 is more expansive, being reported from several regions in Asia such as China, Indonesia, Vietnam, and the Philippines and Latin America such as Ecuador, Honduras, Mexico, and Brazil (Sheehan *et al.*, 2009). Streptococcal disease outbreaks in tilapia cultured in Indonesia have been caused mostly by non-hemolytic isolates of *S. agalactiae* (Taukhid & Purwaningsih, 2011; Lusiastuti *et al.*, 2014).

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Information on the genetic diversity of bacteria is indispensable to understand the cause of disease and develop specific methods for disease prevention and management (Qazi *et al.*, 2020). Genetic variation of this bacterium has been examined using a multilocus sequence typing (MLST) approach to genetically differentiate piscine and bovine *S. agalactiae* (Lusiastuti *et al.*, 2013). Pulsed-field gel electrophoresis (PFGE) and MLST method have also been employed to distinguish between *S. agalactiae* strain and the genetic variation. The bacterium has a vast spectrum of hosts, including humans, cows, and fish (Delannoy *et al.*, 2013).

Bacterial virulence is closely related to the presence and function of virulence genes. Virulence genes, which include adhesins, invasins and immune-evasions, protein and enzyme regulators associated with the metabolism of the cell surface, have been identified as essential virulence factors of *S. agalactiae* (Kayansamruaj *et al.*, 2014; Kayansamruaj *et al.*, 2015; Delannoy *et al.*, 2016).

In this study, *S. agalactiae* obtained from tilapia farms in Indonesia were screened for the presence of virulence genes. The relationship between virulence genes and the degree of virulence and pathogenicity to nile tilapia were also evaluated.

MATERIALS AND METHODS

Fish and Bacteria Samples

Isolates included in this study originated from Papua (NP104O/A; NP105O/B), Gorontalo (NMbO/J), South Borneo (S01-196-16/C), Jambi (SG01-16/I), Central Java (NK1/F), and West Java (NB002O/D; N14G/E; NT010/G; N2O/H). These were isolated originally from nile tilapia during streptococcosis outbreaks. Isolates were inoculated on brain heart infusion agar (BHIA; Oxoid Ltd, UK) and incubated for 24 hours at 28°C-30°C. Koch's postulate test was performed on all isolates to confirm their disease role before identifying the virulence gene and conducting pathogenicity test.

Hemolytic activity was tested by growing bacteria in blood agar base media with 5% v/v sheep blood, incubated for 24-48 hours at 37°C. Confirmation test for bacterial isolates were also conducted by using API 20 Strep System commercial kit (bioMérieux Industry, Hazelwood, USA). Species specific identification of *S. agalactiae* was performed using the method in Lusiastuti *et al.* (2013) by modification of temperature optimization in cycle condition using primer sequence agal-I 5'ATAAGAGTAATTAACACATGTTAG-3' (forward) and agal-II 5'ACTTCGGGTGTTACAAAC-3' (reverse) with amplification target 1,250 bp (Macrogen, Korea).

Nile tilapia strain Nirwana III weighing 17 ± 1.4 g were obtained from tilapia and Common Carp Stock Enhancement and Development Centre, Wanayasa, West Java, Indonesia. The fish were acclimatized in the laboratory for two weeks. To confirm that experimental fish were not carriers of *S. agalactiae*, kidney tissues were collected from five randomly selected fish and tested using the PCR method with universal primers agal-I and agal-II as described above. The experimental procedures and treatment of tilapia (*Oreochromis niloticus*) in this study have been approved by the Indonesian local authorities (Animal Care and Use Committee, IPB University, Indonesia).

Identification of Virulence Gene

Identification of virulence genes of *S. agalactiae* was performed using methods described in Kayansamruaj *et al.* (2014) and Ye *et al.* (2011) (Table 1). Each strain of bacteria was cultured in BHIA medium. The bacteria were suspended in 200 μL TE buffer (10 mmol/L Tris–HCl, 1 mmol/L EDTA; pH 8.0) and then heated on thermomixer 98°C for 10 min, and centrifuged on 14,000 g for 10 min. DNA concentration was measured with spectrophotometer Nano Drop™ (Thermo Scientific, USA). DNA obtained was stored as aliquots at -20°C until used.

The PCR was performed in thermal cycler T-personal (Biometra, UK), using Cycling conditions included an initial denaturation step of two minutes at 94°C, 25 cycles of one minute at 95°C for denaturation, one minute at 55°C for annealing, and 90 seconds at 72°C for elongation/extension and final elongation/extension at 72°C for five minutes. Electrophoresis of PCR products was conducted using 12 ìL amplicon in 1.5% agarose gel at 120 volts in 1x Tris-acetate-electrophoresis buffer (TAE) [(0.04 mol/L Tris; 0.001 mol/L EDTA; pH 7.8)] for 15 minutes alongside 25 bp DNA Step Ladder marker (Promega, Madison WI USA) and 100 bp DNA ladder (MBI Fermentas).

Challenge Test

The challenges test was initiated by separating the fish into eleven treatment groups. Each fish group was kept in a tank with stocking density of 20 fish/tank. Each strain of *S. agalactiae* bacteria was cultured in a brain heart infusion broth (BHIB) and incubated for 24 h at 28°C-30°C. Fish were challenged by intraperitoneal injection (IP) of 24 hours cultured *S. agalactiae* isolates as much as 0.1 mL of 10^8 CFU/fish of β-hemolytic (NP104O/A, NP105O/B, S01-196-16/C, SG01-16/I) and non-hemolytic (NB002O/D, N14G/E, NK1/F, NT010/G, N2O/H, NMbO/J) isolates and control was injected with 0.1 mL phosphate buffer saline (PBS). Observation of clinical signs and mortality was

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Table 1. Primers used for *S. agalactiae* virulence genes identification

Virulence gene	Gene product	Primer name	Primer sequence (5'–3')	Product length (bp)
cylE	*B-hemolysin cytolysin*	*cylE_F* *cylE_R*	TTCTCCTCCTGGCAAAGCCAGC CGCCTCCTCCGATGATGCTTG	124*
hylB	*Hyaluronate lyase*	*hylB_F* *hylB_R*	TCTAGTCGATATGGGGCGCGT ACCGTCAGCATAGAAGCCTTCAGC	136*
Lmb	*laminin binding protein*	*lmb_F* *lmb_R*	TGGCGAGGAGAGGGCTCTTG ATTCGTGACGCAACACACGGC	105*
bibA	*Immunogenic bacterial adhesion*	*bibA_F* *bibA_R*	AACCAGAAGCCAAGCCAGCAACC AGTGGACTTGCGGCTTCACCC	127*
PI-2b	*pili-2b backbone*	*PI-2b_F* *PI-2b_R*	AGGAGATGGAGCCACTGATACGAC ACGACGACGAGCAACAAGCAC	175*
fbs A	*Fibrinogen-binding protein A*	*fbs A_F* *fbs A_R*	GTCACCTTGACTAGAGTGATTATT CCAAGTAGGTCAACTTATAGGGA	85*
fbs B	*Fibrinogen-binding protein B*	*fbs B_F* *fbs B_R*	TCTGTCCAACAGCCGGCTCC TTCCGCAGTTGTTACACCGGC	144*
Gap	*GAPDH*	*gap_F* *gap_R*	AGACCGATAGCTTTTGCAGCACC GATCCTTGACGGACCACACCG	100*
PI-1	*pili-1 backbone*	*PI-1_F* *PI-1_R*	AACAATAGTGGCGGGGTCAACTG TTTCGCTGGGCGTTCTTGTGAC	102*
Cfb	CAMP factor	*cfbS* *cfbA*	CGACAGCATCACACGAAAAATACA TGACGACCTTTTGGACAAGTAGTAA	900**

Note: * Kayansamruaj *et al.* (2014); ** Ye *et al.* (2011)

performed every hour for the first 24 hours, and afterwards, observations were taken daily for 14 days of the culture period.

Histology

Histology preparation procedures followed the methods described in Genten *et al.* (2009). Briefly, histology analysis was performed on the liver fixed in 10% neutral buffered formalin for 24 h. The tissue was then dehydrated, embedded in paraffin, and cut into 5 µm thick sections on a rotary microtome (Histo Line MRS 3500), followed by Haematoxylin and Eosin (H&E) staining.

Data Analysis

The study was designed as a completely randomized design experiment. Data obtained were tabulated and analyzed using Microsoft Excel 2013. Effect of treatment analyzed with analysis of variance (ANOVA) with *Minitab Software* version 17. Significant different results were further analyzed with fisher test on a 95% confidence level. Detection of virulence gene and clinical signs analyzed and described qualitatively.

RESULTS AND DISCUSSION

Streptococcus agalactiae is a pathogenic bacterium that can cause disease in various biota. This pathogen has developed multiple strategies to infect a host by involving virulence-related factors, including invasion of the host cell and replication in the host, ultimately leading to cell death (Fittipaldi *et al.*, 2012).

S. agalactiae isolates were examined for the presence of the following virulence genes *B-hemolysin cytolysin* (*cylE*), *Hyaluronate lyase* (*hylB*), *laminin binding protein* (*lmb*), *Immunogenic bacterial adhesion* (*bibA*), *pili-2b backbone* (*PI-2b*), *Fibrinogen-binding protein A* (*fbs A*), *Fibrinogen-binding protein B* (*fbs B*), *GAPDH* (*gap*), *pili-1 backbone* (*PI-1*) and CAMP factor (*cfb*) (Table 1) (Ye *et al.*, 2011; Kayansamruaj *et al.*, 2014). The non-hemolytic group was found to have the following six genes (*hylB*, *bibA*, *fbsA*, *fbsB*, *gap*, and *cfb*) while the β-hemolytic group was found to possess seven viru-

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lence genes (*cylE*, *hylB*, *bibA*, *PI-2b*, *fbs A*, *fbs B*, and *gap*). Isolate SG01-16 (β-hemolytic) differed from the other β-hemolytic isolates as it did not have PI-2b, but had the *cfb* gene. None of the isolates examined in this study found the virulence genes *lmb* and *PI-1* (Table 2).

Based on the presence of virulence genes, these isolates were assigned to three groups referred to as β-hemolytic (A, B, C), β-hemolytic (I) and non-hemolytic (Figure 1). Bacteria of β-hemolytic with code (I) have the same virulence genes as non-hemolytic bacteria, except gene *cylE*. Bacteria of β-hemolytic with code (A, B, C) have the same virulence genes as code (I) bacteria, except *PI-2b* and *cfb* (Figure 1).

The results of this study showed that both *S. agalactiae* hemolytic and non-hemolytic bacteria groups have the same virulence genes. Virulence genes from the adhesion group (*fbsA*, *fbsB*, *bibA*, and *gap*) and invasion group (*hylB* and *cylE*) on β-hemolytic and (*hylB* and *cfb*) on non-hemolytic. While virulence genes *lmb* and *PI-I* were not detected in all isolates. The results of Kayansamruaj *et al*. (2014) showed that *S. agalactiae* had no *lmb* virulence gene. Delannoy *et*

Table 2. Virulence gene of *S. Agalactiae*

Virulence gene	Gene product	Detection gene of *S. agalactiae* strains											
		A	B	C	D	E	F	G	H	I	J	ST-260	ST-23
cylE	*B-hemolysin cytolysin*	+	+	+	-	-	-	-	-	+	-	+	+
hylB	*Hyaluronate lyase*	+	+	+	-	+	+	+	+	+	+	+	+
lmb	*laminin binding protein*	-	-	-	-	-	-	-	-	-	-	-	+
bibA	*Immunogenic bacterial adhesion*	+	+	+	-	+	+	+	+	+	+	+	+
PI-2b	*pili-2b backbone*	+	+	+	+	-	-	-	-	-	-	+	-
fbs A	*Fibrinogen-binding protein A*	+	+	+	-	+	+	+	+	+	-	+	+
fbs B	*Fibrinogen-binding protein B*	+	+	+	-	+	+	+	+	+	+	-	+
gap	*GAPDH*	+	+	+	-	+	+	+	+	+	-	ND	ND
PI-1	*pili-1 backbone*	-	-	-	-	-	-	-	-	-	-	-	-
cfb	CAMP factor	-	-	-	-	+	+	+	+	+	+	ND	ND
	Total of virulence gene	7	7	7	1	6	6	6	6	7	4		

Note: A: NP104O; B: NP105O; C: S01-196-16; D: NB002O; E: N14G; F: NK1; G: NT010; H: N2O; I: SG01-16; J: NMbO. STIR-CD-17, ST 260, serotype 1b; MRI Z1-201, ST-23, serotype 1a (Delannoy *et al*., 2014)

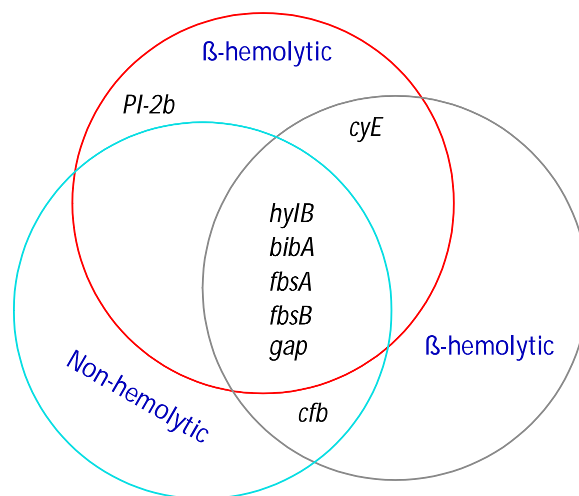

Figure 1. Virulence gene groups of *S. agalactiae* isolates.

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al. (2014) was able to identify 15 virulence genes of *S. agalactiae* and grouped them into three groups, namely adhesin (*fbsA*, *fbsB*, *PI-2b*, *PI-1*, *lmb*, *bibA*), invasin (*cylE*, *cfb*, *hylB*, *gap*), and immune evasin (*pbp1A*, *cspA*). Kannika *et al.* (2017) conducted a study in the correlation of virulence genes on the pathogenicity of *S. agalactiae*, and obtained two different serotypes, IA and III. Eleven virulence genes were found in serotypes Ia, and 13 virulence genes in serotypes III, were positively correlated with bacterial pathogenicity when tested on tilapia. The results of Delannoy *et al.* (2014) also found 15 virulence genes on serotype 1b of and caused high mortality when injected into tilapia.

Pathogenic bacterial infection requires adhesion to host cells, tissue colonization, cellular invasiveness - accompanied by intracellular multiplication, spread to other tissues or persistence. During the entry process, pathogens invade and pass through layers of epithelial cells, extracellular matrix components, and aided by hydrolytic enzymes to degrade host cell molecules and support bacterial replication (Lin *et al.*, 2011). Adhesin virulence gene group is responsible for the initial contact between host pathogens and binding to specific receptors in host epithelium (Rajagopal, 2009). Fibrinogen-binding proteins (*fbsA*) and fibronectin-binding protein (*FnbB*) mediate adhesion of *S. agalactiae* with fibronectin then *Fnb* forms a complex tetramer structure and invasion (Yi *et al.*, 2014).

The invasion gene group functions in the process of bacterial entry into the host cell. It spreads throughout the body, bacteria access in initiating the infection process. For example, hyaluronate lyase (*hylB*) is a virulence factor that helps pathogens break through biophysical barrier of the host tissue with enzymatic degradation of hyaluronan and chondroitin sulfate (Wang *et al.*, 2014). *β-hemolysin/cytolysin* gene known as *cylE* is an important toxin in the bacterial infection process, inducing an inflammatory response that contributes to neurological symptoms (Rajagopal, 2009). Immune evasion is used to avoid the host response mediated by the host's immune system, such as serine proteinase (CspA). Its functions are to cut fibrinogen and chemokines and disrupt the mobilization of neutrophils and phagocytic cells to phagocytosis of *S. agalactiae* bacteria (Fittipaldi *et al.*, 2012). Results of observations of the invasion group gene showed *hylB* and *cylE* were observed on β-hemolytic and *hylB* and *cfb* on non-hemolytic.

Tilapia injected with β-hemolytic group (A, B, C, and I) showed no significant difference in the mean time to death or mortality, and 100% mortality was achieved around 9.3-11.8 hours post-injection (hpi). While the non-hemolytic group (E) caused 53.3%-86.6% mortality after 14 days post-injection (dpi) (Table 3).

Injected tilapia showed clear signs of *S. agalactiae* infection. Both hemolytic and non-hemolytic isolates showed clinical signs such as melanosis, slow response, anorexia, ocular opacity, gasping, erratic, C-shape, and whirling. Different clinical signs were observed between isolated groups, mainly the absence of exophtalmia and purulens on fish injected by β-hemolytic isolates. Symptoms of purulence and exophthalmia were not found in the fish injected with β-hemolytic bacteria because the bacterial attack pattern was acute and causes death in a short time. Clinical symptoms of the fish infected with *S. agalactiae* are similar to Amal & Zamri-Saad (2011) description.

Table 3. Mortality and mean time to death after challenge test of *S. agalactiae* isolates

S. agalactiae isolates		hemolytic activity	Number of fish	Mortality (%)	Mean time to death (hour)
(A)	NP104O	β-hemolytic	60	100 ± 0.00^{a}	11.12
(B)	NP105O	β-hemolytic	60	100 ± 0.00^{a}	10.35
(C)	S01-196-16	β-hemolytic	60	100 ± 0.00^{a}	11.77
(D)	NB002O	non-hemolytic	60	53.33 ± 7.64^{f}	207.00
(E)	N14G	non-hemolytic	60	83.33 ± 2.89^{b}	165.65
(F)	NK1	non-hemolytic	60	$80 \pm 5.00b^{c}$	166.5
(G)	NT010	non-hemolytic	60	$70 \pm 8.66d^{e}$	193.14
(H)	N2O	non-hemolytic	60	73.33 ± 7.64^{cd}	182.73
(I)	SG01-16	β-hemolytic	60	100 ± 0.00^{a}	9.30
(J)	NMbO	non-hemolytic	60	63.33 ± 10.41^{e}	207.16
(K)	Control	PBS	60	0	0

Note: Different superscript letters at the mortality parameter indicate significantly different results within the treatments ($P < 0.05$)

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Livers from tilapia injected with *S. agalactiae* β-hemolytic and non-hemolytic exhibited most hepatocytes were larger than usual and had vacuoles in the cytoplasm that pushed the nucleus to the edge due to degeneration. The parenchyma's large blood vessels thoroughly appear dilated with the lumen filled with blood cells (congestion). Sinusoidal spaces appear to expand due to capillary congestion in the multifocal parenchymal area (Figure 2A and 2B).

Hepatocyte degeneration and congestion of liver sinusoids in response to bacterial infections indicate a detoxification process that impacts tilapia liver dysfunction (Laith *et al*., 2017). The number of empty vacuoles in the cytoplasm will interfere with biochemical reactions. *S. agalactiae* infection in tilapia liver causes vacuolalization of hepatocytes with changes in fat in response to reduced blood flow due to congestion (Suwannasang *et al*., 2014). Periarterial Lymphoid Sheath (PALS) white pulp dominated by T lymphocytes responds to immunity which involves activating cells against infection and destroying infected cells. Depletion in germinal centers in the secondary lymphoid organs where mature B cells proliferate, resulting in reduced production of antibodies against antigens.

CONCLUSION

Streptococcus agalactiae, isolated during outbreaks of streptococcocis in cultured tilapia in Indonesia, carry different combinations of virulence genes. The result showed that β-hemolytic bacteria has seven of ten detected genes, while non-hemolytic strain has six of ten detected genes. Bacteria with more virulence genes showed higher pathogenicity when injected into fish.

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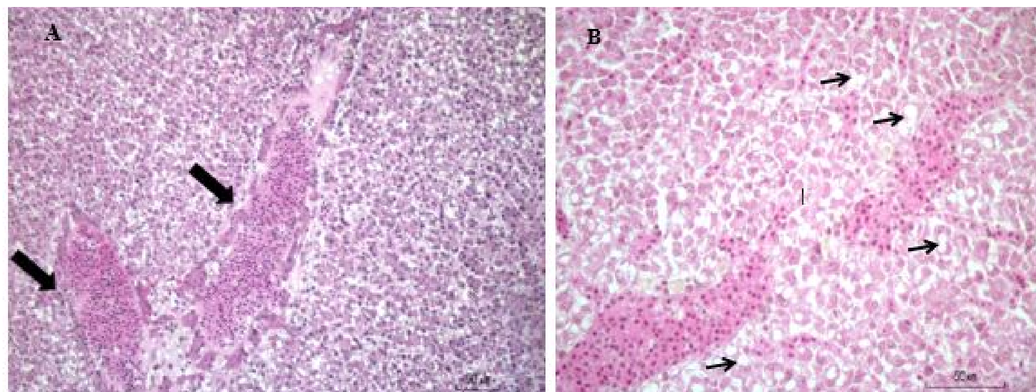

Figure 2. Histopathology of liver. Congestion of the capillary (A) and blank vacuoles in the cytoplasm of hepatocytes (arrow), capillary congestion in the multifocal parenchymal area (head arrow) (B).

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