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ORIGINAL ARTICLE

Severity and prognostic assessment of the endotoxin activity assay in biliary tract infection

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Abstract
Background Acute cholangitis and cholecystitis (AC) often progress to severe septic conditions. We evaluated the endotoxin activity assay (EAA) for assessment and prediction of the severity of AC.
Methods We retrospectively reviewed 98 patients diagnosed with AC. We divided them into low (<0.4) and high (≥0.4) groups based on EAA values.
Results Endotoxin levels showed no correlation with EAA values. Serum C-reactive protein (8.57 vs. 5.23 mg/dl, $P = 0.02$), procalcitonin (2.45 vs. 0.48 ng/ml, $P = 0.004$), and the positive culture rate of blood (50% vs. 15%, $P < 0.001$) were significantly higher in the high group than in the low group. Platelet counts were significantly lower in the high group than in the low group (23.9 vs. 13.5 10^4/ml, $P = 0.004$). The ratio of patients with a Japanese Association for Acute Medicine disseminated intravascular coagulation score ≥4 (32% vs. 14%, $P = 0.032$) was significantly higher in the high group than in the low group. There was a significantly higher percentage of patients with a severe grade of AC in the high group than patients with a mild or moderate grade (32% vs. 15%, $P = 0.05$).
Conclusions Endotoxin activity assay is useful for assessment and early prediction of septic conditions due to AC.

Keywords Cholangitis · Endotoxin · Endotoxin activity assay · Sepsis

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Introduction

The pathogenesis of acute cholangitis is biliary infection associated with partial or complete obstruction of the biliary system. This is caused by various etiologies, including choledocholithiasis, benign and malignant strictures, biliary-enteric anastomotic malfunction, and indwelling biliary stent malfunction. In acute cholangitis and acute cholecystitis (AC), an increase of intraductal pressure often causes damage to bile canaliculi, which readily leads to the spillage of purulent bile into the blood stream. Infected bile flows directly into the veins through a cholangiovenous shunt, which causes bacteremia and endotoxemia [1]. Therefore, acute biliary tract infection progresses from local biliary infection to systemic inflammatory response syndrome (SIRS), because of spillage of pathogens or pathogen associated molecules. Advanced disease then leads to sepsis with or without disseminated intravascular coagulation (DIC) and organ dysfunction.

In 2005, the first guidelines for the management of AC, "Evidence-based clinical practice guidelines for the management of acute cholangitis and cholecystitis" (JGL) [2], were published in Japan. In 2007, another set of guidelines, "Tokyo guidelines for the management of acute cholangitis and cholecystitis" (TG07) [3], were published and accepted as the global standard criteria for the diagnosis and severity assessment of the disease. A revised version of the guidelines (TG13) [4–6], was also published. In TG07,

the requirement for biliary drainage is determined after assessing the severity of AC. Based on this viewpoint, there has been concern for a delay in decisions and the start of drainage because of inappropriate assessment [7, 8]. For more precise prediction of the rapid deterioration of biliary tract inflammation, the establishment of new biomarkers, which can be used for the early assessment of the disease severity, is required.

Endotoxemia is often observed when biliary tract infection progresses to a severe pathological condition of sepsis [9]. For the measurement of endotoxin in blood, a turbidimetric assay using limulus amebocyte lysate (LAL assay) has been widely used. However, the usefulness of this assay in biological samples is limited [10]. Recently, another method for rapid measurement of blood endotoxin levels within 2 h, the endotoxin activity assay (EAA), was developed, and its usefulness is widely accepted [11–13]. Marshall et al. examined endotoxin levels measured by the EAA and found that EAA levels correlate well with disease severity and mortality [11]. Currently, the EAA is mainly used for critically ill patients after admission into the intensive care unit. For patients with AC, early examination of endotoxin levels before intensive care unit admission may be useful for an early decision regarding treatment strategy.

In this study, we measured EAA levels in patients with biliary tract infection at the early stage of disease progression. We also examined the relevance of EAA levels for magnitude of activated neutrophils by endotoxin and evaluated its usefulness as a prediction marker of disease severity.

Patients and methods

Patients who were hospitalized for AC at our department were evaluated for inclusion in this study. They also fulfilled at least two of the diagnostic criteria for systemic inflammatory response syndrome (SIRS) on admission. We excluded patients who were less than 18 years old. The severity of AC was also assessed according to JGL. In TG07, "moderate stage" is phrased as "acute cholangitis that is not respond to the initial medical treatment and not accompanied by organ dysfunction". At a clinical site, it is difficult to judge whether immediate biliary drainage is necessary or not. When AC progresses rapidly, assessment of the disease severity after waiting for the results of conservative treatment is not practical. For this reason, JGL is more commonly used for the disease severity assessment. In this study, we used JGL for the severity assessment and the patients defined as "intermediate" or "severe" AC were all hospitalized and biliary drainage was conducted within 24 h.

Patients' clinical and microbiological data and blood examination data were prospectively collected. We examined the baseline characteristics of age, sex, white blood cell (WBC) count, C-reactive protein (CRP) levels, procalcitonin (PCT) levels, endotoxin levels measured by the LAL assay, EAA levels, and bacterial culture of blood and bile samples on admission. At the time of the EAA, blood samples were drawn and whole blood was sent for blood culture. For analysis, laboratory data were used from the time point closest to when the EAA was performed.

The Japanese Association for Acute Medicine DIC (JAMM DIC score) [14] and the sequential organ failure assessment score (SOFA score) were recorded within 24 h after admission. Additionally, the duration of the JAAM DIC was calculated from electrical medical records.

The study was approved by our institution ethics committee, and informed consent was obtained from all patients.

EAA

The principal of EAA has been described in detail previously [15]. The assay was conducted according to the manufacturer's instructions (Spectral Diagnostics, Toronto, Canada). Whole blood samples of patients were drawn through an indwelling arterial line into ethylenediaminetetraacetic acid (EDTA)-anticoagulant tubes. Five hundred samples were added into empty endotoxin-free tubes (aliquot tubes) and a lipopolysaccharide (LPS)-max tube containing 2.3 ng of *Escherichia coli* endotoxin. After 10 min incubation at 37°C, 40 μl samples from the aliquot tube were added into tube 1 and to tube 2, and a 40 μl sample from the LPS-max tube was added into tube 3. Tubes 2 and 3 contain 2.6 μg/ml endotoxin specific anti-lipid A monoclonal antibody, luminol reagent and zymosan, while tube 1 contains luminol reagent and zymosan, without anti-endotoxin antibody. Tubes were incubated at 37°C for 14 min with gentle shaking. During the incubation, an endotoxin-anti-endotoxin antibody complex was formed, opsonized with complement proteins and primed neutrophils in the blood to enhance their respiratory burst in response to zymosan in the assay reagent. Then oxidants were detected by using luminometer (Berthold Detection Systems, Pforzheim, Germany). Tube 1 was used as a negative control showing basal activity of neutrophils and Tube 3 was used as a positive control showing activity of maximally stimulated neutrophils. EAA levels are expressed as relative values between 0 (lowest) and 1 (highest) according to the following equation; EAA level = (Tube 2 – Tube 1)/(Tube 3 – Tube 1).

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences II for Windows version 11.0J (SPSS II) (Chicago, IL, USA). A descriptive analysis

was first conducted on the entire population. Patients were then stratified according to their EAA results into the following groups: the low (L) group (EAA levels <0.4) and the high (H) group (EAA levels ≥0.4). All values are expressed as median and standard deviation (SD). Data were analyzed by the Kruskal–Wallis test, the Mann–Whitney *U*-test, the χ^2 test, and Fisher's exact test.

Results

We retrospectively reviewed 98 collected consecutive cases of acute biliary tract infection who were admitted between June 2011 and August 2012. The characteristics of patients are shown in Table 1. A total of 95 patients was diagnosed as having acute cholangitis and three patients were diagnosed as having acute cholecystitis according to the TG. Because all our patients had urgent and early billiary drainage performed within 24 h after admission, they had the severity of AC classified according to the JGL: 26 had mild, 51 had moderate, and 21 had severe severity. All patients had urgent endoscopic retrograde cholangiopancreatography (ERCP) performed within 24 h after admission and received initial medical treatment, including antibiotic therapy.

Endotoxin levels measured by the LAL assay were mostly below the detection limit and there was no correlation with EAA levels (Fig. 1).

The mean values of the variables assessed in each group are shown in Table 2. The WBC count was not significantly different between the two groups (P = 0.499). CRP (P = 0.02) and PCT levels (P = 0.004) were increased in the H group compared with the L group. There were no significant differences in the prothrombin time international normalized ratio and fibrin and fibrinogen degradation products between the two groups. However, platelet counts were significantly lower in the H group than in the L group (P = 0.004).

Results of the bacterial culture of blood and bile samples are shown (Tables 3,4). Blood culture was done in the samples from all the patients, while bile culture was conducted in only 55 among 98 patients, since there was the problem of contamination and we could not obtain enough volume of bile samples for bacterial culture in some cases.

The positive culture rate of blood was 28% in all patients. The percentage of distribution of bacterial organisms was 20% for Gram-negative organisms and 8% for Gram-positive organisms (Fig. 2). The positive culture rate of blood was significantly higher in the H group than in the L group (50% vs. 15%, $P < 0.001$). Table 3 shows the distribution of bacterial organisms in blood culture and associated EAA levels. Twelve patients had Gram-negative organisms and five patients were positive for Gram-positive organisms in the H group.

On the other hand, the positive rate of bile culture was 82% in 55 patients. Table 4 shows the distribution of bacterial organisms in bile culture and associated EAA levels. There were no significant correlations between EAA levels and the positive rate of bile culture (90% vs. 76%, P = 0.173) (Fig. 3). In contrast, we found a significant correlation between the disease severity and the results of bile culture (100% vs. 73%, P = 0.016) (Fig. 4). Namely, the positive rate of bile culture was higher in the patients with higher severity of AC.

The ratio of patients with a JAAM DIC score of ≥4 (P = 0.032) was significantly higher and the duration of DIC (6 vs. 2.5 days, P = 0.006) was significantly longer in the H group than in the L group. The ratio of patients with a SOFA score of ≥5 (P = 0.038) was significantly higher and hospital stay (P = 0.008) was significantly longer in the H group than in the L group.

The percentage of patients with a JAAM DIC score of ≥4 (62% vs. 9%, $P < 0.001$) was significantly higher, the percentage of patients with a SOFA score of ≥5 (P < 0.001) was significantly higher, and hospital stay (P = 0.003) was significantly longer in patients with a more severe grade of AC than in patients with a mild or

Table 1 Characteristics of patients

No. patients	98
Age (range)	69.5 (52–85)
Gender (Male : Female)	67:31
Cholangitis : Cholecystits	95:3
Disease severity	
Mild	26
Moderate	51
Severe	21

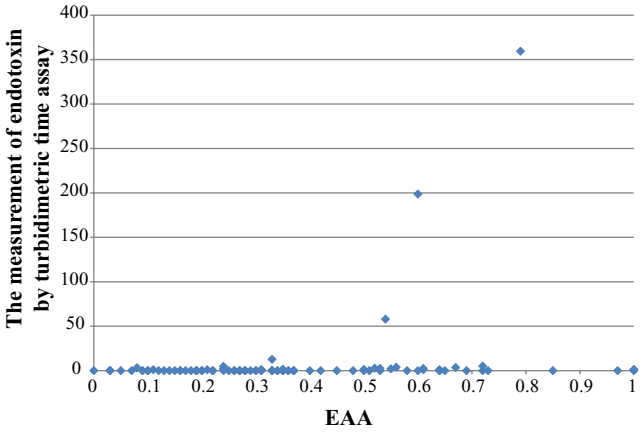

Fig. 1 Endotoxin levels measured by the limulus amebocyte lysate (LAL) assay were mostly below the detection limit and there was no correlation with endotoxin activity (EA) values (r^2 = 0.055, P = 0.02)

Table 2 Characteristics of patients in two groups

Variables	EAA level		*P*-value
	Low: 0–0.39	High: ≧ 0.4	
	(*n* = 64)	(*n* = 34)	
Age (range)	70 (52–85)	69 (54–81)	0.352
Gender (Male : Female)	43:21	24:10	0.457
Inflammatory response			
WBC (/μl)	9350 ± 3918	8900 ± 6231	0.499
CRP (mg/dl)	5.23 ± 6.58	8.57 ± 6.67	0.060
Procalcitonin (ng/ml)	0.48 ± 9.07	2.45 ± 13.5	0.004
Positive rate of blood cultures	15% (10/64)	50% (17/34)	<0.001
Coaglation factor			
Platelet (104/ml)	23.9 ± 7.70	13.5 ± 6.60	0.001
PT-INR	1.16 ± 0.24	1.24 ± 0.41	0.088
FDP-E (μg/ml)	9.90 ± 24.9	15.0 ± 26.7	0.063
JAAM DIC score	1 ± 1.79	2 ± 2.23	0.030
≧4	14% (9/64)	32% (9/34)	0.032
SOFA score	2 ± 1.93	2 ± 2.00	0.071
≧5	6% (4/64)	21% (7/34)	0.038
Disease severity			
(Mild and Moderate: Severe)	54:10	23:11	0.050
Hospitalization (Days)	10 ± 11.1	15 ± 23.9	0.008

CRP C-reactive protein, *EAA* endotoxin activity assay, *FDP-E* fibrin degradation product E, *JAAM DIC* Japanese Association for Acute Medicine disseminated intravascular coagulation, *PT-INR* prothrombin time international normalized ratio, *SOFA* sequential organ failure assessment, *WBC* white blood cell count

moderate grade (Table 5). The ratio of patients with EAA levels ≥0.4 in the severe grade of the JGL was significantly higher than that in patients with a mild or moderate grade (32% vs. 15%, $P = 0.05$) (Fig. 5). The positive culture rate of blood was higher in patients with a more severe grade than in patients with a mild or moderate grade (57% vs. 19%, $P = 0.001$) (Fig. 6).

Discussion

The TG are the accepted global standard criteria for assessment of severity of AC. Basically, severity of AC is categorized as follows both in TG and JGL; "severe stage": require organ support due to AC associated with organ dysfunction, "moderate stage": should be treated by immediate drainage and "mild stage": can be treated with conservative cure. In JGL, specific standard values for severity assessment are indicated about "moderate stage". In TG07, "moderate stage" is described as a stage that requires immediate drainage without organ dysfunction, which is a similar definition to JGL. However, specific standard values are not indicated in TG07 and the moderate stage is defined as "acute cholangitis that does not respond to the initial medical treatment and is not associated with organ dysfunction". According to this definition, the severity of the disease can be assessed only after evaluating the results of conservative treatment. It is not suitable for the early assessment of the possibility of rapid worsening. For this reason, we use JGL in daily clinical practice, which can be used for early assessment of the disease. In this research, we analyzed the correlation between EAA levels and the severity of the disease based on JGL. In a newly published guideline, TG13, specific standard values are used for severity assessment as in JGL. For this reason, we think TG13 will be a useful and practical guideline for clinicians in addition to clinical evaluation standards. However, because AC sometimes rapidly progresses to a fatal state, a marker that can correctly evaluate the severity of the patient's condition at an earlier time point is urgently required.

Infected bile flows directly into veins through a cholangio-venous shunt, which causes bacteremia and endotoxemia. Gram-negative bacteria are the main causative organisms in AC, and endotoxemia is commonly observed [16–18]. Severe sepsis and subsequent systemic organ failure due to severe AC are caused by blood infection of bile bacteria [9]. This results in a state of bacteremia and severe sepsis, which may lead to a fatal outcome. For this reason, detection of endotoxemia is important for the rapid evaluation of disease severity.

The LAL assay is widely used for measurement of endotoxin in blood. Blood culture is also used for the detection of bacteremia, but the results of a positive culture rate are not usually high. Additionally, both methods require time to obtain results, and their clinical usefulness is limited because of low sensitivity and possible false positive results. The EAA has been developed and used as an alternative to the LAL assay [15]. In the MEDIC study, the prevalence of Gram-negative infection was shown to be significantly high in the high group (EA ≥ 0.6) and low in the low group (EA < 0.4) [11]. In our study, the positive culture rate of blood was significantly different between an EAA level < 0.4 and an EAA level ≥ 0.4. Therefore, we analyzed the correlation of positive culture rate of blood with various severity criteria based on this categorization of two groups.

However, the diagnostic criteria of updated Tokyo Guidelines (TG13) [4–6], endotoxin levels are excluded from the criteria for severity assessment, because there is no correlation between the severity of acute biliary tract infection and endotoxin levels. Indeed, the routinely used LAL assay has many uncertainties and is not reliable for blood samples. In our study, we found out that endotoxin levels measured by EAA were not correlated with those of the LAL assay, and most of the LAL assay results were below the detection

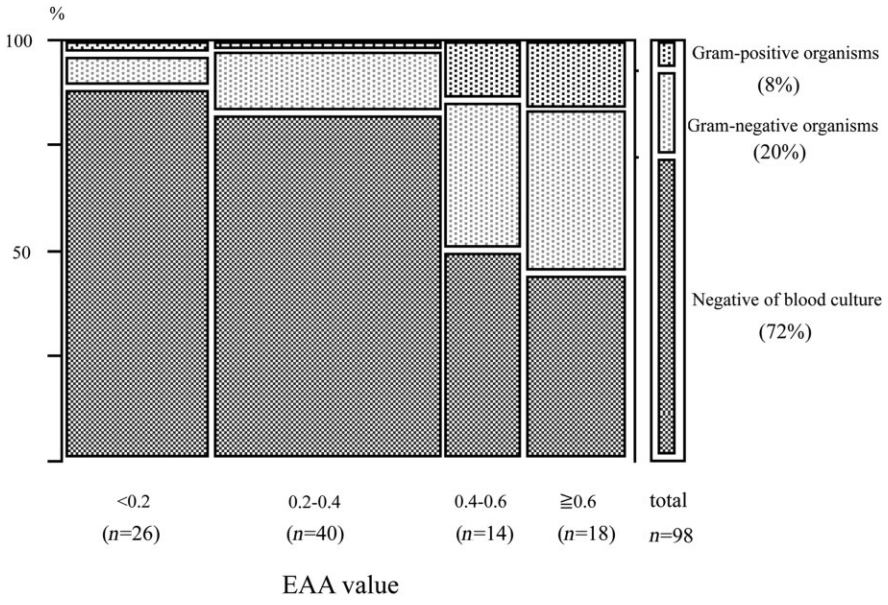

Fig. 2 Results of blood culture according to endotoxin activity assay (EAA) values. The positive culture rate of blood was significantly different between EAA values <0.4 and those >0.4. The positive culture rate of blood was significantly higher in the H group than in the L group (50% vs. 15%, $P < 0.001$)

Table 3 Endotoxin activity assay (EAA) and organisms in blood culture

Variables	EAA level			
	Low: 0–0.39		High: ≧ 0.4	
	(n = 10)		(n = 17)	
	Gram negative infection		**Gram negative infection**	
	Escherichia coli	4	*Klebsiella pneumoniae*	5
	Klebsiella pneumoniae	2	*Escherichia coli*	4
	Pseudomonaus aeruginosa	1	*Enterobacter cloacae*	2
	Klebsiella oxitoca	1	*Klebsiella oxytoca*	1
	Gram positive infection		**Gram positive infection**	
	Enterococcus faecalis	1	*Staphylococcus sanguinis*	1
	Clostridium perfringens	1	*Staphylococcus haemolyticus*	1
	Staphylococcus aureus (MRSA)	1		
	Staphylococcus cohnii-cohnii (MRS)	1		
	Enterococcus faecium	1		

limit, independent of disease severity and blood culture results. In contrast, EAA levels showed a significant correlation with the severity of cholangitis based on the JGL and were also correlated with positive culture rate of blood culture. Yaguchi et al. also reported that endotoxin activity levels are higher in sepsis patients with Gram-negative infection and the EAA was useful for the diagnosis of sepsis [18]. The present study suggested that the EAA is practical for evaluating biliary tract infection. The EAA levels reflect blood infection and magnitude of activated neutrophils by endotoxin, as well as the severity of AC. On the other hand, the positive rate of bile culture was 82% in 55 patients. However, the number of samples analyzed in this study was not large enough and further studies are needed to examine the correlations among the bacterial culture results, EAA levels and the severity of disease in more detail.

There are several reports showing that the organ dysfunction score (SOFA score) is higher in patients with high EAA levels [12, 13, 18, 19]. In our study, platelet counts were significantly lower and the JAAM DIC score [14] was significantly higher in patients with high EAA levels than in those with low EAA levels. Furthermore, hospital stay was significantly longer in patients with high EAA levels than in those with low EAA levels. Based on these results, EAA may be useful for the stratification and prediction of disease severity of AC.

In our study, the positive rate of bile culture was 82% in 55 patients. However, the number of samples analyzed in this study was not large enough and further studies are needed to examine the correlations between the bacterial culture results, EAA levels and the severity of disease in more detail.

Table 4 Endotoxin activity assay (EAA) and organisms in bile culture

Variables	EAA level				$n = 55$
	Low: 0–0.39		High: ≧ 0.4		
	($n = 29$)		($n = 19$)		
	Gram negative infection		**Gram negative infection**		
	Escherichia coli	8	*Escherichia coli*	6	
	Citrobacter frendii	5	*Klebsiella pneumoniae*	4	
	Enterobacter cloacae	3	*Enterobacter cloacae*	2	
	Klebsiella oxitoca	2	*Pseudomonas aeruginosa*	1	
	Pseudomonaus aeruginosa	1	*Citrobacter frendii*	1	
	Klebsiella oxytoca	1			
	Gram positive infection		**Gram positive infection**		
	Enterococcus faecium	3	*Enterococcus faecium*	2	
	Enterococcus faecalis	2	*Enterococcus faecalis*	2	
	Enterococcus avium	1	*Enterococcus species*	1	
	Enterococcus species	1			

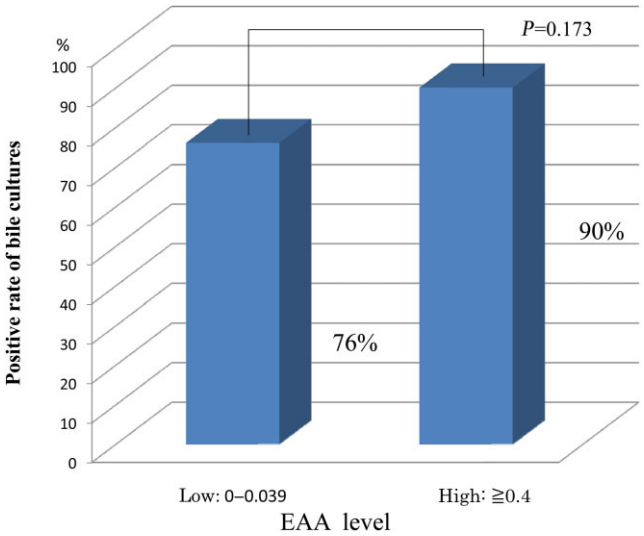

Fig. 3 There were no significant correlation between endotoxin activity assay (EAA) level and the positive rate of bile culture (90% vs. 76%, $P = 0.173$)

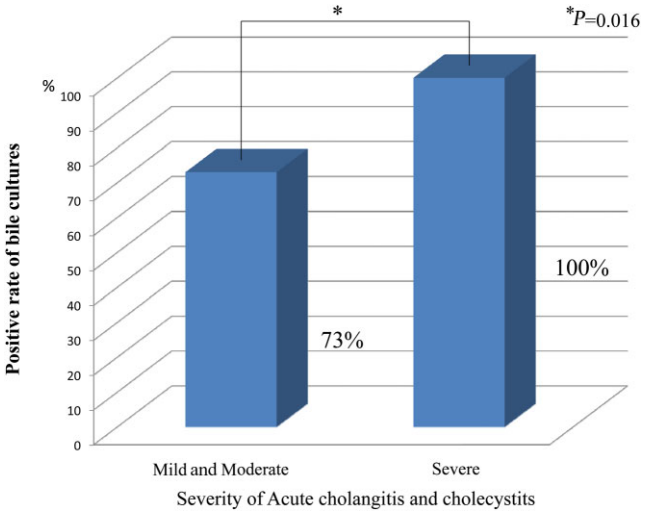

Fig. 4 The positive culture rate of bile was higher in patients with a severe grade, according to *The Guidelines for Acute Cholangitis and Cholecystitis Based on Scientific Evidence (JGL)*, than in patients with a mild or moderate grade (100% vs. 73%, $P = 0.016$)

Endotoxin is a component of the membrane of Gram-negative bacteria. EAA levels measure the magnitude of biological response to endotoxin. Occasionally, high EAA levels were seen in the samples in which Gram-negative bacteria were not detected by blood culture. Furthermore, in our study, five samples were shown to be Gram-positive bacterial infection by blood culture with high EAA levels (≥0.4). There is also the possibility that peptide glycans from Gram-positive cocci might be detected by toll-like receptor 2 and subsequently stimulate monocytes and neutrophils. Several patients with low EAA levels (<0.4) showed positive results in blood culture. Most of those cases were categorized as a mild or moderate grade of severity. For anatomical reasons, bacteria in the biliary tract can easily enter neighboring blood vessels. We speculate that in the abovementioned patients, bacteria that temporarily flowed into the blood stream were eliminated by phagocytosis of immune cells activated by inflammatory cytokines, such as tumor necrosis factor-α, interleukin-1, and interleukin-6, resulting in prevention of progressive infection and deterioration of the disease. Because the EAA uses neutrophils in samples for the detection of endotoxin-anti-endotoxin complexes, it may be affected by the neutrophilic conditions of patients to some extent. Individual differences in the neutrophil response to pathogen-associated molecular patterns, substances activating the

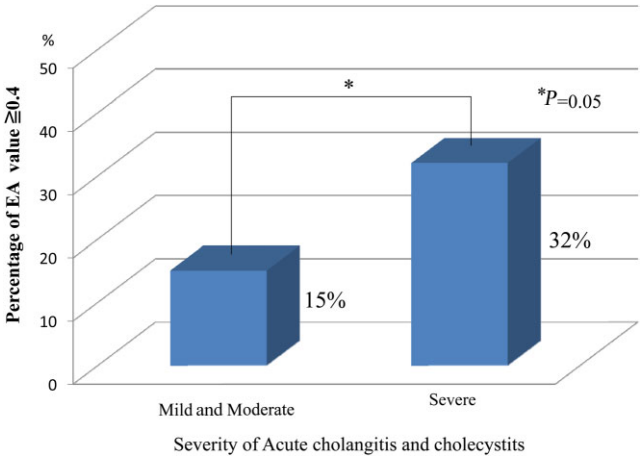

Fig. 5 Percentage of high endotoxin activity assay (EAA) values according to the severity of biliary infection. There was a significantly higher percentage of patients with a severe grade in the H group than in the L group (50% vs. 15%, $P = 0.05$)

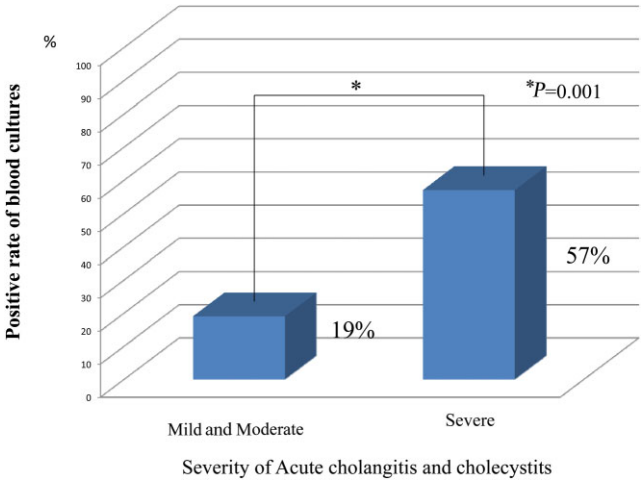

Fig. 6 The positive culture rate of blood was higher in patients with a severe grade than in patients with a mild or moderate grade (57% vs. 19%, $P = 0.001$)

Table 5 Characteristics of patients in severity

Variables	Mild and moderate	Severe	*P*-value
	(*n* = 77)	(*n* = 21)	
JAAM DIC score			
≧4	9% (7/77)	62% (13/21)	<0.001
SOFA score			
≧5	4% (3/77)	38% (8/21)	<0.001
Hospitalization (Days)	10 ± 17.3	15 ± 17.1	0.003

JAAM DIC Japanese Association for Acute Medicine disseminated intravascular coagulation, *SOFA* sequential organ failure assessment

innate immune system, may affect EAA values. Therefore, the combination of the EAA levels and other biomarkers may improve the performance of disease severity prediction.

In conclusion, rapid and precise assessment of disease severity is critically important for the improvement of patient outcomes of AC diagnosed based on the TG. We also show that EAA levels may be a useful marker for that purpose.

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Conflict of interest None declared.

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