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Original Article

Epidemiological and pathobiological profiles of *Clostridium perfringens* infections: review of consecutive series of 33 cases over a 13-year period

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Abstract: Background: Although *Clostridium perfringens (C. perfringens)* is well known as the causative agent of several forms of enteric disease, precise epidemiological and pathobiological aspects are still unknown. Methods: We retrospectively reviewed the culture results of samples collected in our hospital from 2001 through 2013. In addition, for the detection and toxinogenic typing of *C. perfringens*, polymerase-chain-reaction amplification (PCR)-based rapid analysis was performed in 6 cases using DNA extracted from paraffin-embedded tissues. Results: A total of 35 samples from 33 cases were positive for *C. perfringens*, representing an incidence of 0.017% (35/205, 114). Among 33 patients, 21 patients manifested sepsis and 7 patients had bacteremia. One of the septic cases was complicated by fatal intravascular hemolysis and thus, the prevalence was estimated at 3.0% among *C. perfringens* infections (1/33). The direct causative disease or state for C. perfringens infection was identified in 18 patients: surgery or intervention for cancers, 8 patients; chemotherapy for cancer, 2 patients; surgery or intervention for non-neoplastic disease, 6 patients; liver cirrhosis, 3 patients, etc. PCR-based toxinogenic typing of *C. perfringens* detected the alpha-toxin gene only in tissue from a patient who died of massive hemolysis; none of the toxin genes could be amplified in the other 5 cases examined. Conclusions: The prevalence of overt *C. perfringens* infection is low, but upon detection, infected patients should be carefully monitored for fatal acute hemolysis caused by type A *C. perfringens*. Furthermore, PCR-based rapid detection of *C. perfringens* and toxinogenic typing by archival pathological material is applicable as a diagnostic tool.

Keywords: *Clostridium perfringens*, fatal acute hemolysis, alpha-toxin, polymerase-chain-reaction amplification

Introduction

Clostridium perfringens (*C. perfringens*), an anaerobic, gram-positive bacillus, is a normal inhabitant of the human bowel and genitourinary tract [1]. This bacterium is well known as a causative agent of several forms of enteric disease, including food poisoning and fatal enterotoxemia [2, 3]. There are five types of *C. perfringens*, A, B, C, D and E, classified according to the production of four major exo-toxins (alpha, beta, epsilon and iota). The most commonly encountered type A (alpha-toxin-producing type) strain causes gas gangrene (myonecrosis) and food-borne illness in addition to enterotoxemia in humans [4]. Type B (alpha-, beta-, and epsilon-toxin positive) and type D (alpha- and epsilon positive) strains are the causative agents of fatal enterotoxemia in animals and occasionally in humans [4]. Type C (alpha- and beta-toxin positive) also causes fatal enterotoxemia in humans [2, 5]. Type E (alpha- and iota-toxin positive) has rarely been isolated in humans and thus, its pathogenicity remains unclear [2]. These species are sporadically isolated from samples, but such results are often interpreted as false positives due to environmental contamination.

The diagnosis of *C. perfringens* can be routinely made by microbiological isolation and characterization methods, including bacterial culture

Table 1. Overall clinical profiles of 33 patients

Clinical factors	Categories	Summary measure
Age	< 55 years	1 (3.0%)
[mean, 75.6 years; 41-98 years]	55 ≤, < 65 years	4 (12.1%)
	65 ≤, < 75 years	8 (24.2%)
	75 ≤, < 85 years	15 (45.5%)
	85 years ≤	5 (15.2%)
Sex	Male	19 (57.6%)
	Female	14 (42.4%)
Samples (35 samples)	arterial blood	6 (18.2%)
	venous blood	19 (57.6%)
	bile	6 (18.2%)
	peritoneal fluid	3 (9.1%)
	surgical wound (pus)	1 (3.0%)
Clinical Diagnosis	cholangitis/cholecystitis	6 (18.2%)
	urinary tract infection	5 (15.2%)
	peritonitis	5 (15.2%)
	pneumonia	5 (15.2%)
	sepsis	4 (12.1%)
	liver abscess	2 (6.1%)
	enterocolitis	2 (6.1%)
	infectious endocarditis	1 (3.0%)
	surgical site infection	1 (3.0%)
	myonecrosis	1 (3.0%)
	unknown	1 (3.0%)
Entry site	respiratory tract	5 (15.2%)
	urinary tract	5 (15.2%)
	bowel	6 (18.2%)
	biliary tract	8 (24.2%)
	surgical wound	1 (3.0%)
	unknown	8 (24.2%)
Clinical condition	Sepsis (*SIRS)	21 (63.6%)
	Bacteremia (without SIRS)	7 (21.2%)
	Others	5 (15.2%)
Trigger	operation or intervention	15 (45.5%)
	cancer	9 (27.3%)
	non-neoplastic disease	6 (18.2%)
Immunocompromised factor	diabetes	4 (12.1%)
	liver cirrhosis	3 (9.1%)
	chemo (radiation) therapy	2 (6.1%)
	interstitial pneumonia	2 (6.1%)
	hemodialysis	2 (6.1%)

*SIRS, systemic inflammatory response syndrome.

and biochemical analysis [2, 6]. However, isolation from intestinal contents does not necessarily lead to the diagnosis of enterotoxemia, as this micro-organism is a normal inhabitant of the bowel even in healthy humans [7]. The further toxinogenic typing of *C. perfringens* is performed by the mouse neutralization test (MNT) after the isolation of the micro-organism [6-8]. However, since classic culture procedures detect only live microorganisms, those conventional techniques are not applicable for the retrospective detection and further toxinogenic genotyping of nonviable bacteria, such as those in formalin-fixed, paraffin-embedded (FFPE) tissues. In this context, during the past few decades, polymerasechain-reaction amplification (PCR) techniques have been applied to detect and to type *C. perfringens* [2, 7]. PCR is a well-accepted, rapid and sensitive technique for the detection of microbial pathogens, and moreover, is efficient under circumstances in which bacteria are present in low numbers.

In the current study, we retrospectively examined the epidemiological, clinical, and pathological aspects of the patients from whose cultures *C. perfringens* was isolated, including a case manifesting fatal acute hemolysis.

Materials and methods

Samples and patients' profiles

We retrospectively reviewed the bacteriological records of the patients' samples, including blood, bile, cystic content and pleural/peritoneal fluid submitted and cultured in the Division of Bacteriology, Central Clinical Laboratory,

Table 2. Detail clinical profiles of 33 patients in the current series

	Age	Gender	diagnosis	clinical condition	entry site	sample	diabetes	compromised state	cancer	operation	DNA available	prognosis
1	41	M	Peritonitis	sepsis	bowel	peritoneal fluid	-	P.O.[4] 3 weeks	rectum	+		alive
2	55	F	UTI[1]	bacteremia	urinary tract	V[2]	-	chemotherapy	uterine body	-		alive
3	57	M	Enterocolitis	sepsis	bowel	V	-	chemoradiation therapy	lung	-		alive
4	59	M	Peritonitis	sepsis	unknown	V	+	LC[5]	-	-		alive
5	61	F	Peritonitis	no bacteremia	bowel	peritoneal fluid	-	P.O. 1 day	cecum	+	+	alive
6	67	M	Cholangitis	sepsis	biliary tract	bile	-	IP[6], CHF[7]	-	+		alive
7	69	F	UTI	sepsis	urinary tract	V	-	-	-	+		alive
8	70	M	Cholangitis	no bacteremia	biliary tract	bile	+	-	-	+		alive
9	71	M	Pneumonia	bacteremia	respiratory tract	V	-	-	lung	-		alive
10	71	M	Liver abscess	sepsis	biliary tract	V	+	LC	liver	+	+	dead
11	73	M	Cholecystitis	sepsis	biliary tract	bile	-	P.O. 3 weeks	-	+	+	alive
12	73	F	Liver abscess	sepsis	biliary tract	bile	-	-	bile duct	+	+	dead
13	73	F	Unknown	bacteremia	unknown	V	-	-	uterine body	-		unknown
14	75	M	Sepsis	sepsis	unknown	V	-	-	rectum	+		alive
15	75	F	Cholangitis	sepsis	biliary tract	bile	-	-	-	-		alive
16	76	F	Peritonitis	sepsis	unknown	A[3], V	-	LC	liver	+		dead
17	76	F	Pneumonia	bacteremia	respiratory tract	V	-	-	-	-		alive
18	76	M	Peritonitis	no bacteremia	bowel	peritoneal fluid	-	HD[8]	-	-		dead
19	76	F	Myonecrosis	sepsis	bowel	A	-	-	rectum	-		alive
20	77	F	Cholangitis	sepsis	biliary tract	V	-	P.O. 3 weeks	gallbladder	+		alive
21	78	M	SSI[9]	no bacteremia	wound (surgcal site)	pus	-	IP	-	-		alive
22	78	F	IE[10]	bacteremia	unknown	A	-	IE	-	+		alive
23	79	M	Cholangitis	no bacteremia	biliary tract	bile	-	P.O. 5 days	pancreas	+	+	alive
24	81	M	Sepsis	sepsis	unknown	A	-	CPA[11] on arrival	-	-		dead
25	82	M	Sepsis	sepsis	unknown	V	-	P.O. 2 months	pancreas	+		alive
26	83	F	Sepsis	sepsis	unknown	V	-	P.O. 7 days	stomach	+	+	alive
27	84	M	UTI	bacteremia	urinary tract	A	-	-	-	-		alive
28	84	M	UTI	bacteremia	urinary tract	V	-	-	-	-		alive
29	90	F	enterocolitis	sepsis	bowel	V	-	-	-	-		alive
30	93	M	UTI	sepsis	urinary tract	V	-	-	colon, bladder	-		alive
31	96	M	Pneumonia	sepsis	respiratory tract	V	-	HD	melanoma	+		alive
32	97	F	Pneumonia	sepsis	respiratory tract	V	-	-	-	-		alive
33	98	F	Pneumonia	sepsis	respiratory tract	A, V	+	-	-	-		alive

[1]UTI, urinary tract infection; [2]V, verous blood; [3]A, arterial blood; [4]P.O., post operative state; [5]LC, liver cirrhosis; [6]IP, Interstitial pneumonia; [7]CHF, congestive heart failure; [8]HD, Hemodialysis; [9]SSI, surgical site infection; [10]IE, infectious endocarditis; [11]CPA, cardiopulomary arrest.

Table 3. PCR primers used in this study

Gene	Primer sequences 5' to 3'	Primer position	Primer concentration (μM)	Product size	Reference
cpa/alpha	GCTAATGTTACTGCCGTTGACC	1438-1457	0.5	324	26
	TCTGATACATCGTGTAAG	1762-1743			
cpb/beta	GCGAATATGCTGAATCATCTA	871-891	0.36	196	27
	GCAGGAACATTAGTATATCTTC	1067-1046			
etx/ipsilon	GCGGTGATATCCATCTATTC	227-246	0.46	655	28
	CCACTTACTTGTCCTACTAAC	882-862			
iA/iota	ACTACTCTCAGACAAGACAG	275-294	0.52	446	29
	CTTTCCTTCTATTACTATACG	721-701			
cpe/enterotoxin	GGAGATGGTTGGATATTAGG	439-458	0.34	233	30
	GGACCAGCAGTTGTAGATA	672-650			

Saitama Medical Center, Jichi Medical University, between 2001 and 2013. The identification of *C. perfringens* had been performed by Rap ID kit (ANA II System, Remel Inc. KS, U.S.A.) after culture in HK semi-solid medium and on Brucella HK agar plates (Kyokuto Pharmaceutical Industrial. Ltd., Tokyo, Japan) [6-8]. This retrospective review detected 33 patients positive for *C. perfringens* (**Table 1**). Next, the medical records of these 33 patients were reviewed for age, gender, clinical profile, underlying disease, past medical events, radiographic records, and laboratory data. Each of the patients was classified into one of three categories: i) sepsis, defined as systemic inflammatory response syndrome (SIRS) caused by infection; ii) bacteremia, without SIRS; and iii) other, defined as no SIRS or bacteremia, according to the guidelines of the ACCP/SCCM Consensus Conference Committee (**Table 1**) [9]. This study was approved by the Institutional Tissue Committees and written informed consent from each patient was obtained prior to the molecular analysis described below.

Samples used for PCR

Out of 33 cases, FFPE tissues were available in 9 cases, and informed consent for the molecular analysis was obtained from 6 patients (Cases 5, 10-12, 23, and 26). These six cases included resected specimens for cancers of the cecum (Case 5), the pancreas (Case 23), the stomach (Case 26), cholecystitis (Case 11) and liver abscesses obtained by autopsy (Cases 10, 12). All the samples had been fixed in 15% buffered formalin (pH 7.4) for 24 to 48 hrs prior to embedding in paraffin. The paraffin blocks had been stored for periods between 6 months and 11 years. Microscopic examination of the tissue sections from all, but one case (case 12) revealed no identifiable gram-positive bacilli morphologically suggestive of *C. perfringens*.

Preparation of samples for PCR

Three 10-μm-thick sections were prepared from each paraffin block, using a new microtome blade for each block, and transferred into a microcentrifuge tube. The sections were deparaffinized by two 30-min incubations in 1 mL xylene at 60°C followed by centrifugation at 1.5×10^4 *g* for 5 min. The resulting pellets were washed 3 times in 1 mL of absolute ethanol. In parallel, pure cultures of *C. perfringens* type A (NCTC8798) carrying alpha- and enterotoxin gene, type B (GTC15078) carrying alpha-, beta-, and epsilon-toxin gene and type E (GTC15081) carrying alpha- and iota-toxin, were used as positive controls for PCR [2, 7].

Total DNA was extracted using the DNeasy blood and tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. In brief, each sample was placed in 340 μL of buffer ATL with 25 μl of proteinase K (50 mg/mL), and reaction mixtures were incubated at 56°C overnight. The supernatants were obtained by a brief centrifugation, mixed with 400 μL buffer AL, and incubated at 70°C for an additional 20 min. After incubation, 400 μL of ethanol was added, and the mixture was passed through a QIAamp spin column. DNA was eluted from the column with 100 μL of buffer AE.

Primers for PCR

The oligonucleotide primers specific for *C. perfringens* loci were synthesized as previously described [10]. Sequences and locations of the

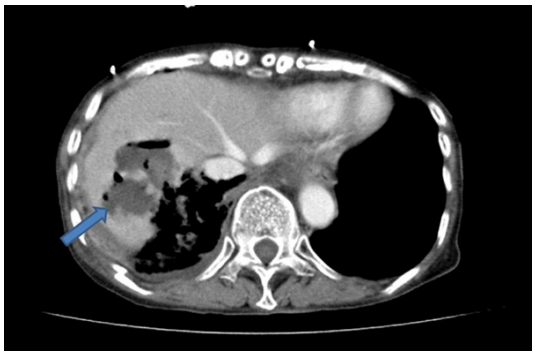

Figure 1. Radiographical imaging of the liver showing gas-containing (pseudo)cystic lesion in the liver found in Case 12.

primers for genes encoding alpha-, beta-, epsilon-, iota-toxin or enterotoxin are shown in **Table 3**, and the predicted product sizes were 324, 196, 655, 446 and 233 bp, respectively [10].

PCR procedures

PCR was performed in a 50-μL scale composed of 2 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 10 mM KCl, 0.2 mM of each dNTPs, 0.2 μM of each primer, 20 ng of DNA, and 1.25 U *TaKaRa Ex Taq* Hot Start Version (TAKARA BIO INC. Otsu, Shiga, Japan). The reaction was performed in a thermal cycler (GeneAmp PCR system 9700; Applied Biosystems, CA, USA) for 35 cycles, with each cycle consisting of 1 min at 94°C, 1 min at 53°C, and 1 min at 72°C, and cycle was followed by 10 minutes at 72°C. PCR products and molecular size markers (TrackIt 100 bp DNA Ladder, Invitirogen CA, USA) were gel-electrophoresed in a 2.0% agarose gel and visualized after staining with 0.5 μg/mL ethidium bromide.

Results

Culture samples and patients' profiles

All culture samples collected since 2001 have been monitored in our hospital, which is a tertiary care center with 608 beds. Among the total of 205, 114 samples, *C. perfringens* were isolated from 33 patients (0.017%), of whom 21 were categorized with "sepsis" (SIRS caused by infection), 7 with "bacteremia" (infection that was positive for *C. perfringens* in blood culture, but was not complicated by SIRS), and 5 with "other" (**Table 1**). Clinicopathological details of each patient are presented in **Table 2**. The number of patients presenting with isolates of *C. perfringens* generally increased with the patient's age (younger than 55 years, 1 patient; 55-64 years, 4 patients; 65-74 years, 8 patients; 75-84 years, 15 patients; 85-97 years, 5 patients). Twenty eight patients (84.8%) were > 65 years old; mean age and standard deviation was 75 ± 12 years (range, 41-98 years). There were 19 male and 14 female patients. The samples were submitted from various wards: emergency room (8 patients), gastrointestinal medicine (8 patients), gastrointestinal surgery (8 patients), cardiovascular medicine (2 patients), cardiovascular surgery (2 patients), gynecology (2 patients), urology (2 patients) and neurosurgery (1 patient).

As the most common underlying disease or state, surgery or intervention for cancer of the liver, of the hepatobiliary tract, or of the pancreas was present in 6 patients: operation or intervention for pancreatic cancer, 2 patients; bile duct cancer, 1 patient; gallbladder cancer, 1 patient; transarterial infusion for hepatocellular carcinoma, 2 patients (both died). Other surgeries included those for cancer of the gastrointestinal tract in 3 patients. Chemo (-radiation) therapy was performed in 2 patients: one case each for uterine and lung cancer. Surgery or intervention for non-neoplastic disease was performed in 6 patients: cholecystectomy for cholelithiasis (Case 6), nephrectomy for pyelonephritis (Case 7), partial hepatectomy for benign cyst (Case 8), ileocecal resection for ischemic enteritis (Case 11), colectomy for perforation (Case 18), and mitral valve replacement (Case 22). Five patients were conservatively treated: lung cancer (Case 9), uterine cancer (Case 13), brain abscess (Case 17), rectal cancer (Case 19), and colon cancer (Case 30). Viewed from the entry site, infections were classified as follows: urinary tract, 5 patients; bowel, including bacterial translocation, 6 patients; upper respiratory tract, 5 patients; biliary tract, 8 patients; surgical site, 1 patient; unknown sites, 8 patients.

In addition to the 15 patients who presented with infections after surgical intervention, 13 patients were classified with an immunocompromised or related condition: diabetes mellitus, 4 patients (Cases 4, 8, 10, 38); chemo (radiation) therapy for cancer, 2 patients (Cases 2, 3); liver cirrhosis, 3 patients (Cases 4, 10,

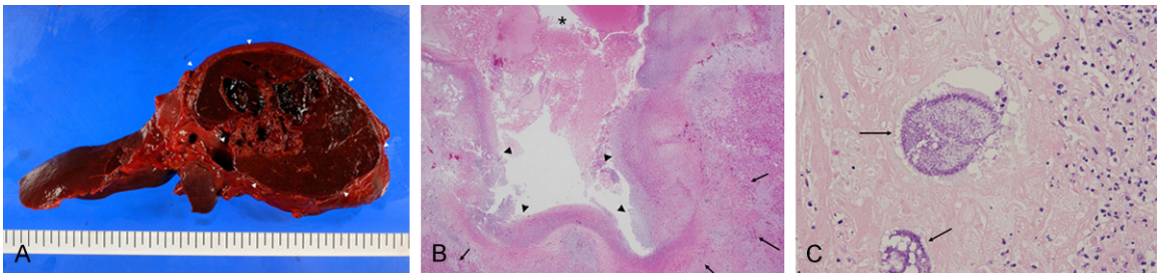

Figure 2. A. Gross appearance of the liver obtained from the autopsy in Case 12. Cut surface revealed pseudocystic and partially necrotic lesion of 8 cm in the maximum dimension (arrow heads). B. Histological features of pseudocyst wall of the liver in Case 12. Lumen was filled with blood clot and fibrin (*). Inner surface was covered by inflammatory exudate and necrotic material (arrow heads), and surrounded by viable hepatic tissue (arrows). C. Around the inner necrotic area, colonies of gram-positive bacillus were observed (arrows).

16); interstitial pneumonia, 2 patients (Cases 6, 21); and hemodialysis, 2 patients (Cases 18, 31). A total of five patients (Cases 8, 10, 12, 13, 16) died of sepsis. The other 28 patients recovered from the infection. Among 21 patients with sepsis, one patient (Case 12) was a typical case complicated by fatal acute hemolysis whose profile is presented below.

Report of a typical case

A 73-year-old female, who had a past history of pancreatoduodenectomy for bile duct carcinoma at 59 years old, was admitted to our hospital with chief complaint of general malaise and jaundice. She had suffered from fracture of the right 6th and 7th ribs by traffic accident 7 days prior to admission and had been managed conservatively.

The patient appeared confused at the time of admission, presenting with a temperature of 36.0°C, blood pressure 120/80 mmHg, and pulse rate of 110 bpm. Abdominal examination showed tenderness in the upper abdomen.

Laboratory findings showed severe anemia, massive hemolysis, liver dysfunction, inflammatory reaction, and renal dysfunction: Clinical laboratory assays yielded the following: white blood cell count, 8500/mm^3; red blood cell count, 210000/mm^3; hemoglobin, 4.8 g/dL; hematocrit, 1.8%; platelet count, 440000/mm^3; albumin, 2.4 g/dL; total bilirubin, 10.34 mg/dL; direct bilirubin, 5.84 mg/dL; NH_3, 1420 μg/dL (normal range; 30-86); cholinesterase, 118 U/L (normal range; 170-445); aspartate aminotransferase, 706 U/L; alanine aminotransferase, 168 U/L; lactate dehydrogenase, 6138 U/L (normal range; 110-220); creatine kinase, 998 U/L (normal range; 6-142); alkaline phosphatase, 740 U/L; γ-glutamyltransferase, 12 U/L; C-reactive protein, 9.21 mg/dL; urea, 77 mg/dL; creatinine, 1.44 mg/dL.

An abdominal enhanced computed tomographic (CT) scan revealed a low-density area with gas formation in the right lobe of the liver (**Figure 1**). A diagnosis of liver abscess with possible rupture into the abdominal cavity was made.

Despite the fluid replacement therapy, the patient's hemodynamic condition was unstable. A CT-guided percutaneous transhepatic drainage from the liver abscess was performed. After the drainage in spite of intensive treatment with massive blood transfusion, the patient expired twelve hours after admission. A culture of the liver abscess obtained through drainage tube revealed the presence of *C. perfringens* and *Klebsiella oxytoca*. Autopsy was performed and the main finding was an 80-mm liver abscess with perforation to the abdominal cavity (**Figure 2A**). Cavity was filled with blood clot. Microscopically, inner surface was covered by inflammatory exudate and necrotic material, and surrounded by viable hepatic tissue (**Figure 2B**). In the necrotic material, colonies of gram-positive bacillus were observed (**Figure 2C**). Moreover, microscopic aggregates of red blood cells with fibrin were remarkable in renal tubuli, and scattered fresh thrombi also were found in the glomeruli, suggesting disseminated intravascular coagulation.

PCR amplification yielded the products of expected sizes for *cpa* (alpha-toxin, 324 bp), *cpb* (beta-toxin, 196 bp), *etx* (epsilon-toxin, 655 bp), *iA* (iota-toxin, 446 bp) and *cpe* (enterotoxin,

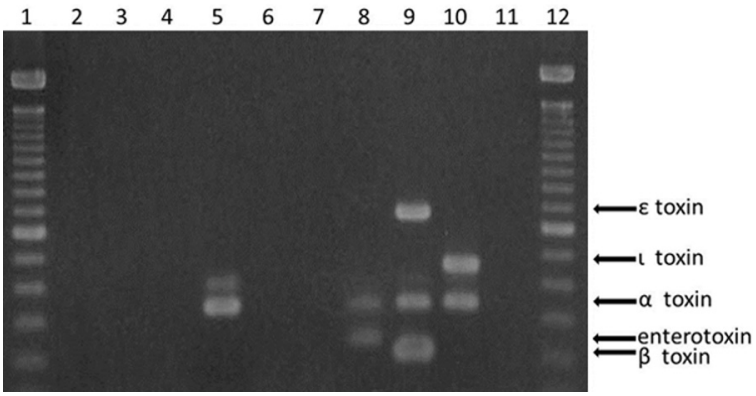

Figure 3. Agarose gel electrophoresis of PCR amplicons obtained using formalin-fixed paraffin-embedded colon tissues from 6 cases (lanes 2-7, Case 5, 10-12, 23, and 25, respectively). *Clostridium perfringens* type A (NCTC8798) carrying alpha- and entero-toxin gene (lane 8), and type B (GTC15078) carrying alpha-, beta-, and epsilon-toxin gene (lane 9), type E (GTC15081) carrying alpha- and iota-toxin gene (lane 10), negative control (lane 11), and molecular size marker (lanes 1 and 12). Lane 5 (Case 12) showed amplified product of *Clostridium perfringens* type A-specific alpha-toxin gene.

233 bp) genes in the appropriate positive controls (**Figure 3**) [10]. Among the FFPE-derived samples obtained from 6 patients, the alpha-toxin gene was amplified only from the Case-12 specimen; none of the other toxin genes were amplified from any of the samples.

Discussion

Patients who contract *C. perfringens* infections exhibit various clinical symptoms including alimentary intoxication, necrotizing enteritis, liver abscess, gas gangrene in the soft tissue, and septic shock [11, 12].

Approximately, 0.5-2.0% of all isolates from blood cultures of septic patients are described as clostridial species, and among them, *C. perfringens* is the most frequently identified microorganism, accounting for 20-50% [11, 13]. Clostridial sepsis often shows very poor prognosis, owing to the life-threatening combination of shock and acute massive hemolysis [14, 15]. Massive hemolysis is rare complication of *C. perfringens* infection, and thus, its prevalence has not been estimated accurately. Among 33 cases from whose samples *C. perfringens* was cultured in our hospital during the past 13 years, only one case manifested massive intravascular hemolysis, suggesting a prevalence of 3.0%. However, the mortality rate of clostridium-induced massive hemolysis is extremely high, ranging from 70 to 100% [1, 16]. Alpha-toxin, mostly in *C. perfringens* of type A, is a causative toxin with phospholipase C activity, capable of hydrolyzing lecithin into phosphorylcholine and diglyceride. As the cell membrane consists of lipoprotein complexes containing lecithin, alpha-toxin leads to the destruction of cell membrane and subsequently to cell death. The sequelae include necrosis or hemolysis, depending on the tissues involved [16]. The appropriate treatments for *C. perfringens* sepsis are early diagnosis, extirpation of the focus of infection, prompt initiation of antibiotic treatment, and hyperbaric oxygen therapy [1, 16]. Unfortunately, early diagnosis of *C. perfringens* infection is difficult, because in the early stages, infected patients only manifest non-specific inflammatory symptoms and the slight gas formation as observed in the infected lesions by imaging studies [11].

In Case 12, the diagnosis could not be made when the patient was alive due to the fulminant clinical course. The results of the blood culture and hepatic drainage revealed *C. perfringens* as the pathogen after the patient died. In addition to this patient, another four patients died, giving an infection-related mortality rate of 15.2%.

The presence of clostridial species in blood cultures are explained by multiple factors, including transient bacteremia (often from an unknown source), trauma, myonecrosis, surgery-related bowel leakage, and occasionally retrograde biliary infections [12, 13], consistent with the examples of (emphysematous) cholecystitis, cholangitis, and liver abscess seen in our series. Cancer and immunosuppressive conditions are the main underlying diseases or states for *C. perfringens* bacteremia or sepsis [11]. In the present study, the most frequent underlying conditions were cancers of the hepatobiliary system and the pancreas (6 cases). Bacteremia caused by *C. perfringens* often occurs in immunocompromised patients [12], and indeed, 15 of 33 patients (45.5%) in the current series were of this status, including

being post-operative, post-intervention, or undergoing chemo (radiation) therapy for malignancy. Additional presumed risk factors noted include advanced age, diabetes mellitus, or past history of surgery for biliary and pancreatic cancers (e.g., for placement of choledocho-jejunostomy) as previously described [12].

The identification of bacterial type requires laborious analysis in addition to the routine procedures of bacterial culturing [6-8]. In the current retrospective study, residual samples from patients or bacterial colonies from growth on agar were not available, since most of the samples had been collected more than a year previously. Therefore, we tried to detect the presence of the individual genes encoding each type of exotoxin, as well as the enterotoxin gene, by PCR with total DNA extracted from FFPE tissues. Furthermore, we aimed to find a difference among several categories of pathological lesions comprising gross liver abscesses (2 cases), which were presumed to have locally higher amounts of bacteria, and cancers of the stomach, cecum, and pancreas (one case each) as well as cholecystitis, which were expected to have locally lower levels of bacteria. We detected the presence of the alpha-toxin-encoding gene in only one case (Case 12), obtained from a patient who showed liver abscess and acute hemolysis, confirming that the disease pathogen was type A.

To our knowledge, there is only one previous report that describes the identification of toxinogenic types of *C. perfringens* from human FFPE tissues. In that work, *C. perfringens* type C in a patient with emphysematous gastritis, and type B or D in a liver with gas gangrene was detected [2]. Case 12 in our series corresponds to a case complicated by fatal massive hemolysis, which we believe represents the first report of *C. perfringens* type A detected in FFPE human liver tissue.

Although negative PCR results cannot prove the absence of *C. perfringens*, the amount of DNA was below the level of detection threshold of this experimental setting. Since the technique presented here is a qualitative rather than quantitative, it was not possible to estimate the amount of *C. perfringens* in the samples. Moreover, considering the characteristics of the samples, which were FFPE tissues, and the presumed uneven distribution of bacteria within infected tissue, quantification may be impossible. Nonetheless, we speculated that the *C. perfringens* was locally concentrated and present at higher levels in the liver abscess of Case 12, since that specimen's DNA was not expected to be well preserved: the tissue was partially necrotic, and the tissues had been left in the paraffin block for a period longer than for the other FFPE specimens. Therefore, the lesion presumably contained locally concentrated *C. perfringens*, which could have been an inducer of the massive hemolysis observed in this patient. Although the diverse pathobiological mechanisms of *C. perfringens* remain to be elucidated, this method (PCR-based detection of *C. perfringens* using DNA extracted from archival FFPE tissues obtained from a pathology laboratory) could be widely utilized for diagnosis and determination of toxinogenic types of *C. perfringens* in clinical specimens.

In conclusion, *C. perfringens* was detected in routine bacterial culture of patient samples at a frequency of 0.017%. Among the clostridial-infected patients, fatal massive intravascular hemolysis occurred at 3.0%, possibly due to the presence of a lesion containing localized high-density *C. perfringens*. In addition, determination of toxigenic gene status by PCR with FFPE tissue may facilitate medical treatment strategies.

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Disclosure of conflict of interest

None.

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