Clostridium perfringens and other anaerobes isolated from bile

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SUMMARY *Clostridium perfringens* was isolated from bile in 13 cases of 150 patients examined. The serotypes of *C perfringens* strains isolated from bile and faeces were investigated using antisera to Hobbs' type 1-17. Two or more serological types were often found in a single specimen, but in the same patient the serotypes of *C perfringens* strains isolated from the bile were identical with those from the faeces.

Beta-glucuronidase production in these *C perfringens* serotypes was tested with the API-Strep system. Strains agglutinated with Hobbs' antisera produced β -glucuronidase, but non-agglutinated strains did not.

The presence of bacteria in the bile from gallbladder or duct is thought to be evidence of biliary tract infection. Bacteria isolated from bile collected by duodenal aspiration are often the target of chemotherapy.

There have been many reports on aerobes in the bile, especially Gram-negative rods such as *E coli*, *Klebsiella*, and *Enterobacter*.¹

In this paper, we classified the anaerobes isolated from 150 patients' bile as to species, and in each of the 13 cases of *C perfringens*, the strain was serotyped with Hobbs' antisera. We are interested in whether *C perfringens*, now firmly established as an important cause of food poisoning, also plays a role in gallstone formation. It has been suggested by Maki that β -glucuronidase may catalyse the hydrolysis of bilirubin glucuronide into free bilirubin and glucuronic acid, and that the free bilirubin may then combine with calcium to form insoluble salts.² Bacterial β -glucuronidase was extensively studied in the aerobes *E coli*, *Streptococcus pyogenes*, *Staphylococcus albus*, and *Shigella*,³⁻⁶ but that of anaerobes has not yet been studied. Therefore we investigated β -glucuronidase production in *C perfringens* from bile.

Material and methods

Two hundred and seventy-nine specimens from 150 subjects were received at the Bacteriology Department of the Central Laboratory of Kansai

Medical University Hospital between May 1981 and April 1982. These specimens were cultured both aerobically and anaerobically.

Gifu Anaerobic Medium (GAM) agar (Nissui Co, Tokyo, Japan) with egg-yolk, and Phenyl-ethylalcohol agar (Nikken Co, Kyoto, Japan) were used for anaerobic culture. Horse-blood agar (Eiken Co, Tokyo, Japan) and MacConkey agar (Eiken) were used for aerobic culture. After inoculation of a bile sample, aerobic cultures were incubated for 24 h and anaerobic cultures for 48 h, at 37°C. Anaerobic cultures were grown in an anaerobic culture system (Forma Scientific Co, USA), in which an absolute anaerobic atmosphere can be achieved by evacuation and replacement of the air with a mixture of 80% N₂, 10% H₂, and 10% CO₂.

Bacterial morphology was checked by Gram's stain, and colonies were transferred to two plates of egg-yolk GAM agar. One plate was cultured anaerobically, and the other aerobically. Alpha toxin (lecithinase) production was detected by opalescence around colonies on egg-yolk agar.

The strains from blood agar or egg-yolk plates were typed into the known Hobbs' serotypes 1-17 by slide agglutination tests using heat-stable *C perfringens* type A antisera for diagnosis (Denka-Seiken, Tokyo, Japan).^{7,8} The reference strain of *C perfringens* (GAI 0084) was obtained from Gifu University, Gifu, Japan. Identification of anaerobes was performed with an API-20 A system (API Laboratory Products Ltd, France) with reference to the *VPI Anaerobe laboratory manual*.⁹

For the detection of β -glucuronidase activity,

Table 1 Anaerobes isolated from bile

Species	No of strains
Cocci	
<i>Veillonella parvula</i>	10
<i>Peptostreptococcus anaerobius</i>	7
<i>Peptostreptococcus productus</i>	2
<i>Peptostreptococcus lanceolatus</i>	1
<i>Peptococcus aerogenes</i>	3
<i>Peptococcus anaerobius</i>	1
<i>Peptococcus prevoti</i>	4
<i>Streptococcus intermedius</i>	2
<i>Streptococcus constellatus</i>	3
<i>Streptococcus morbillorum</i>	2
Total	36
Gram-negative rods	
<i>Bacteroides asaccharolyticus</i>	1
<i>Bacteroides melaninogenicus</i>	2
<i>Bacteroides fragilis</i>	3
<i>Bacteroides vulgatus</i>	2
Total	8
Nonspore Gram-positive rods	
<i>Propionibacterium acnes</i>	2
<i>Propionibacterium granulosum</i>	1
<i>Lactobacillus acidophilus</i>	1
<i>Lactobacillus fermentum</i>	1
<i>Lactobacillus</i> sp	2
<i>Bifidobacterium adolescentis</i>	3
<i>Eubacterium lentum</i>	1
<i>Eubacterium</i> sp	1
<i>Actinomyces odontolyticus</i>	1
Total	13
Spore-forming rods	
<i>Clostridium perfringens</i>	13
<i>Clostridium paraprutificum</i>	1
<i>Clostridium limosum</i>	1
Total	15

C perfringens strains were cultured on egg-yolk GAM agar and the colonies suspended in 0.9% saline solution, then poured into the plates of an API-Strep system.

Results

Bile samples were obtained from 150 patients, of which 87 (58%) were culture-positive. Anaerobes were isolated in 41 cases (27%), with a total of 72 strains (Table 1). Among the strains isolated were *Clostridium* (15), *Peptostreptococcus* (10), *Veillonella* (10), *Peptococcus* (8), *Bacteroides* (8), *Streptococcus* (7), *Lactobacillus* (4), *Propionibacterium* (3), *Bifidobacterium* (3), *Eubacterium* (2), and *Actinomyces* (1). As *Veillonella* and *Peptostreptococcus* exist in the normal oral flora, we thought that these bacteria might have been mixed with the bile.

We are particularly interested in *C perfringens*, especially in those strains which produce β -glucuronidase. *C perfringens* was also isolated from bile in 13 cases (Table 2). There was no sex difference in the incidence, but *C perfringens* was mostly isolated from older patients. All of the patients in our study had abdominal pain, fever, and jaundice. The clinical diagnosis of these 13 patients were choledocholithiasis, chronic cholecystitis, gastric cancer with cholelithiasis, cholelithiasis, choledochal cyst, cholangioma, and suppurative cholangitis. Cases SA and JK were urgently sent to our hospital with acute abdominal pain. SA had repeated pains in the right hypochondrium region with fever and jaundice. JK had general fatigue with abdominal pain, chills, and jaundice. FK had congenital choledochal dilation, and had had a choledochoduodenostomy when she was 17 years old. She had abdominal pain, fever, and jaundice caused by the stricture of the bile duct. Four patients, (TN, NM, RI, JK), had choledochal dilation demonstrated by hepatobiliary scintigraphy. Those patients (YS, TS,

Table 2 Cases of *Clostridium perfringens* isolated from bile

Patient	Age (yr)	Sex	Cell dose	Diagnosis	Other isolated organisms
TN*	67	F	+++	Choledocholithiasis	<i>E coli</i> +, <i>K aerogenes</i> +, <i>C freundii</i> (+)
NM	70	F	++	Chronic cholecystitis	<i>E coli</i> ++
IH*	73	M	++	Gastric cancer cholelithiasis	<i>E coli</i> +++ , <i>P mirabilis</i> (+)
YS†	73	M	(+)	Chronic cholecystitis	<i>E coli</i> (+)
FK	34	F	+++	Choledochal cyst	<i>E coli</i> +++
SA*	78	M	++	Cholelithiasis	<i>E coli</i> +, <i>K oxytoca</i> (+), <i>C diversus</i> (+), <i>Enterococcus</i> (+)
TS†	80	M	(+)	Cholangioma	<i>E coli</i> (+), <i>K aerogenes</i> ++, <i>P mirabilis</i> (+), <i>Enterococcus</i> (+), <i>Staph epidermidis</i> (+)
KM†	51	F	(+)	Cholelithiasis	None
RI*	65	F	+++	Choledocholithiasis	<i>E coli</i> +++ , <i>Aeromonas hydrophila</i> ++
HT*	75	M	+++	Choledocholithiasis	<i>E coli</i> +++
SH*	73	F	+++	Choledocholithiasis	<i>E coli</i> +++ , <i>K aerogenes</i> +++ , <i>K oxytoca</i> (+)
KI*	59	M	++	Gastric cancer cholelithiasis	None
JK	71	M	+	Suppurative cholangitis	<i>E coli</i> +++

*Bile pigment stone was isolated.

†Outpatient.

+++ = 10^{8-9} cells/ml.

++ = 10^{6-7} cells/ml.

+ = 10^{4-5} cells/ml.

(+) = 10^{1-4} cells/ml.

Table 3 Serotypes of *Clostridium perfringens* isolated from bile and the faeces

Patient	Source	Serotype no																	Total strains
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17 Non-typed	
TN	Bile											1	3	2					6
	Faeces											1	6	1					8
NM	Bile														9				9
	Faeces														7				7
IH	Bile	6			2														8
	Faeces	2			6														8
YS	Bile		1								2							4	7
FK	Bile				16														16
SA	Bile					6		3											9
	Faeces					10		2											12
TS	Bile																	4	4
KM	Bile						1												1
	Faeces																		0
RI	Bile	7															1		8
	Faeces	5							1						1		1		8
HT	Bile		1																1
SH	Bile				14						5							1	20
	Faeces																		0
KI	Bile																	8	8
	Faeces				1			2		9								7	12
JK	Bile																		7
	Faeces																		0

Italic numerals refer to those serotypes isolated from bile.

Table 4 Enzyme production of *Clostridium perfringens*

Patient	Serotype	HIP	β -GLU	ALP	β -GAL	PYRA	GGA	ESC
TN	12	+	+	+	+	+	-	+
NM	14	+	+	+	+	+	-	+
IH	1	+	+	+	+	+	-	-
	4	+	+	+	+	+	-	-
YS	2	+	+	+	+	+	-	-
"	10	+	+	+	+	+	-	-
"	Non-typed	+	-	+	+	+	-	-
FK	4	+	+	+	+	+	-	-
SA	5	+	+	+	+	+	-	+
	7	+	+	+	+	+	-	+
TS	Non-typed	+	-	+	+	+	-	-
KM	4	+	+	+	+	+	-	-
RI	1	+	+	+	+	+	-	+
"	8	+	+	+	+	+	-	+
"	14	+	+	+	+	+	-	+
"	16	+	+	+	+	+	-	+
HT	2	+	+	+	+	+	-	+
SH	4	+	+	+	+	+	-	-
"	11	+	+	+	+	+	-	-
"	Non-typed	+	-	+	+	+	-	-
KI	4	+	+	+	+	+	-	-
"	8	+	+	+	+	+	-	-
"	10	+	+	+	+	+	-	-
"	Non-typed	+	-	+	+	+	-	-
JK	Non-typed	+	-	+	+	+	-	+

HIP = hippuric acid hydrolase.

β -GLU = β -glucuronidase.

ALP = alkaline phosphatase.

β -GAL = β -galactosidase.

PYRA = pyrrolidonylarylamidase.

GGA = glycylglycylarylamidase.

ESC = β -glucosidase.

KM) with small cell doses (+) of *C perfringens* were outpatients, while all of the others were inpatients. Nine patients (TN, NM, IH, SA, KM, RI, HT, SH, KI) had cholelithiasis demonstrated by ultrasonography of the biliary tract, and seven of them (TN, IH,

SA, RI, HT, SH, KI) were undergoing an elective operation. Bile pigment gallstones were isolated from all seven of the patients.

In the cases with *C perfringens*, other bacteria isolated at the same time were as follows: 11 cases of

E coli, three cases of *K aerogenes*, two cases each of *P mirabilis*, *K oxytoca* and *Enterococcus*, and one case each of *C freundii*, *C diversus*, *Staph epidermidis*, and *Aeromonas hydrophila*.

When we investigated the serotype of these *C perfringens* strains using antisera to Hobbs' type 1 to 17, we found strains of all types except 3, 9, 15 and 17, and in five cases there were untypable strains. In a single specimen two or more serological types were often found.

As soon as colonies of *C perfringens* could be detected in the egg-yolk GAM agar from the bile sample, we obtained a faecal sample from the patient, before administering antibiotics, and tried to isolate *C perfringens*. In the nine cases where faeces were examined, *C perfringens* was isolated from six patients (Table 3). The serotypes of four patients (TN, NM, IH, SA), coincided exactly with the strains isolated from their bile. Extra serotypes 8 and 14 were isolated from the faeces of RI. In the faeces of KI, the serotypes of *C perfringens* differed from those in the bile. The most frequently isolated strains were four cases of type four, and five cases of non-typed strains.

When we examined β -glucuronidase production in *C perfringens* isolated from bile, Hobbs' typed strains were positive and non-typed strains were negative (Table 4).

Discussion

Bacteria are said to play a major role in the formation of bile pigment calcium stones,² and *E coli*, *Enterococcus*, and *C perfringens* have been isolated from gallstones.¹ In this study, we investigated the Hobbs' serotype and β -glucuronidase production in 13 cases of *C perfringens* isolated from bile.

C perfringens grew rapidly on the egg-yolk agar and was easily isolated from the bile specimens. It could be identified by alpha toxin (lecithinase) production on egg-yolk agar, by stormy fermentation with 10% Litmus milk, and by degradation of 1% gelatin, glucose, and lactose.

In the *C perfringens* strains isolated from bile, two or more serological types were often found in a single specimen and their serotypes differed in each patient (Table 3). However, in the same patient the serotypes coincided well with the strains isolated from the faeces (Table 3). Although *C perfringens* has been found in all faecal specimens from healthy persons,¹⁰ we detected the organism also in the bile of our patients.

When the secretion of gastric juice and bile acids was normal, bacteria were not isolated from the bile collected by duodenal aspiration. In our study, *C perfringens* was isolated from biliary infections such as chronic cholecystitis, cholelithiasis, and suppurative

choolangitis, from choledochal cyst, cholangioma, and gastric cancer with cholelithiasis (Table 2). When we examined the effect of pH on the growth of *C perfringens*, we found it grew rapidly above pH 5.0. The growth of *C perfringens* was inhibited by bile acids.¹¹ We think that *C perfringens* grows if the normal acid production is suppressed. Forty years ago, it was reported that *C perfringens* was isolated from patients with lesions of stomach and duodenum.¹²

When we examined the β -glucuronidase production of *C perfringens*, the Hobbs' typed strains produce β -glucuronidase but the non-typed strains did not. It is curious that the Hobbs' typed strains, which cause food poisoning by an endotoxin, all produced β -glucuronidase, as well.

Another β -glucuronidase-producing anaerobe isolated from bile was *Bacteroides fragilis*. This strain was found by Tharagounet *et al.*¹³ The pH range of the bile was reported to be 6.25 to 8.10.¹ The optimal pH of tissue β -glucuronidase is from 4.5 to 5.5, and above 6.0 its enzyme activity is decreased.¹⁴ When we examined the optimal pH of the β -glucuronidase activity in *E coli* and *C perfringens* the former was 6.3, as reported by Buehler *et al.*,³ and the latter was 7.1. *E coli* produced a β -glucuronidase maximum at 7-8 days, while the rough type of *C perfringens* produced it at 1-2 days and the smooth type at 3-4 days, continuing its β -glucuronidase activity for a total of two weeks.

As mentioned above, we think that in the case of *C perfringens* (Hobbs' typed strain) biliary infection, β -glucuronidase has a significant role in the formation of bile pigment gallstones.

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