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Characteristics of patients with *Clostridium difficile* infection in Taiwan

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SUMMARY

The medical records of 84 patients with stool cultures positive for *Clostridium difficile* during the period August 2007 to June 2009 were retrospectively reviewed. A case of confirmed (toxigenic) *C. difficile* infection (CDI) was defined by the presence of symptoms (fever, diarrhoea, abdominal discomfort or distension, ileus) and the presence of toxigenic *C. difficile*. Patients with compatible clinical symptoms and stool cultures positive for non-toxigenic *C. difficile* isolates were defined as probable (non-toxigenic) CDI cases. Of these 84 patients, 50 (59·5%) were diagnosed as confirmed CDI and 34 (40·5%) as probable CDI. Thirteen (15·5%) of the 84 patients died during their hospital stay. Usage of proton pump inhibitors was a significant independent risk factor for CDI (OR 3·21, $P=0·014$). Of the 50 isolates associated with confirmed CDI, seven (8·3%) carried binary toxin genes (*cdtAB*), and six (7·1%) had a deletion in the *tcdC* gene. The mortality rate in confirmed CDI patients with isolates exhibiting deletion in the *tcdC* gene (2/6, 33·3%), those with isolates harbouring binary toxin genes (2/7, 28·6%), and those with isolates containing mutations in *gyrA* (2/7, 28·6%) and *gyrB* (1/2, 50%) was higher than the overall mortality rate (10/50, 20%) in patients with confirmed CDI.

Key words: *Clostridium difficile* infection, clinical characteristics, outcome, Taiwan, toxin genes.

INTRODUCTION

Clostridium difficile is the most important cause of nosocomial diarrhoea [1, 2]. *C. difficile* infection (CDI) can range in severity from asymptomatic colonization to severe diarrhoea with pseudomembranous colitis to death. Patients infected with highly virulent strains [polymerase chain reaction (PCR) ribotypes 027 and 078] have severe clinical symptoms and a poor prognosis [1, 2]. Studies have shown that highly virulent strains may have a tendency towards fluoroquinolone resistance and tend to contain genes for toxin A, toxin B, binary toxin, and a deletion in

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the *tcdC* gene [1, 3]. Several studies have demonstrated that the role of binary toxin in mediating CDI remains controversial [1, 3].

Few molecular epidemiological studies on the presence of toxic genes of *C. difficile* isolates in patients with CDI have been conducted in Taiwan. We recently found dissemination of a predominant *C. difficile* clone in Taiwan. Although some of the isolates (9·9%) contained toxin A/toxin B, binary toxin, and a deletion in the *tcdC* gene, no isolates of ribotypes 027 or 078 were found [4].

Two recent studies on the prevalence and clinical features of CDI in Taiwan demonstrated that 36·4% of patients with CDI had prolonged diarrhoea and that the recurrence rate was 8·1% in southern Taiwan [5, 6]. In addition, both of those studies showed that the incidence of CDI increased during the study period. Those studies, however, did not investigate the association between the severity and outcome of patients with CDI and the presence of toxin genes (toxin A/toxin B and binary toxin) in the isolates.

PATIENTS AND METHODS

Patients and setting

This retrospective study included 84 inpatients aged $\geqslant$18 years with a positive stool culture for *C. difficile* during the period August 2007 to December 2009. Positive cultures were obtained from 79 patients at the National Taiwan University Hospital (NTUH), a 2900-bed tertiary-care hospital, and from five inpatients at the National Cheng-Kung University Hospital (NCKUH), a 1600-bed institution. Clinical information including age, gender, the presence of underlying diseases, clinical presentations, use of drugs (antimicrobial agents, steroids, and anti-peptic ulcer agents) within 30 days of onset of symptoms, laboratory data obtained 2 days before or 1 day after diagnosis, and outcome was collected from medical records.

Definitions

Diarrhoea was defined as $\geqslant$3 unformed stools occurring within 24 h [7, 8]. Fever was defined as a body temperature $\geqslant$38 °C. A case of confirmed (toxigenic) CDI was defined by the presence of gastrointestinal symptoms, such as fever, diarrhoea, abdominal discomfort or distension, or ileus, with a stool culture positive for toxigenic *C. difficile* strains [9]. In our study, patients with compatible clinical symptoms and stool cultures positive for non-toxigenic *C. difficile* isolates were defined as probable (non-toxigenic) CDI cases. CDI-related mortality was defined as patients with confirmed CDI who died as a consequence of CDI during hospitalization. Invasive disease was defined as patients with blood cultures positive (bacteraemia) for *C. difficile*.

Bacterial culture for *C. difficile*

Liquid or semisolid stool samples were inoculated onto cycloserine-cefoxitin-fructose agar (CCFA, BBL Microbiology Systems, USA) [10]. After incubation at 35 °C for 48 h under anaerobic conditions, growth was identified as *C. difficile* on the basis of Gram staining results (large Gram-positive rods), typical odour, and biochemical characteristics by the Vitek anaerobe identification card (ANI) (bioMérieux Inc., France). Isolates of *C. difficile* were frozen at −70 °C in brain-heart infusion broth (BBL Microbiology Systems) and 15% glycerol prior to testing.

Microbiological analysis

A multiplex PCR assay was used for the detection of *tcdA*, *tcdB*, *cdtA*, and *cdtB*, with 16S rDNA as an internal PCR control. All isolates were subjected to *tcdC* gene sequencing [11]. The *gyrA* and *gyrB* genes of the isolates were amplified using the primer couple as described previously [4, 12]. An automated repetitive extragenic palindromic sequence-based PCR (rep-PCR) typing method (DiversiLab; Bacterial Barcodes Inc., USA) was used to determine the DNA fingerprints of the 84 isolates in accordance with the manufacturer's instructions [4, 13]. Similarity of the isolates (rep-PCR types) was determined and interpreted as previously described [4, 13, 14]. PCR ribotypes for isolates of the main rep-PCR types were determined as previously described [13, 15].

Statistical analysis

Clinical characteristics and outcomes of patients with confirmed and probable CDI (two groups) were analysed. The Student's *t* test was used to compare continuous variables between groups and the results are presented as mean ± standard deviation (S.D.). The χ^2 test was used to compare categorical variables between groups. Risk factors with a *P* value $<$0·10 in the univariate analyses were included in a multiple logistic regression model to study the association

between risk factors for CDI and patients' outcomes. All statistical analyses were performed with the statistical package SPSS for Windows version 12 (SPSS Inc., USA). A P value $<0{\cdot}05$ was considered to indicate statistical significance.

RESULTS

Records of 84 patients with positive stool cultures of *C. difficile,* including 79 patients from the NTUH and five from at the NCKUH, during the period August 2007 to December 2009 were reviewed. All of the patients presented with fever and concurrent gastrointestinal symptoms (diarrhoea, abdominal pain, ileus). Of these 84 patients, 50 (59·5%) were identified as confirmed CDI cases and 34 patients were probable CDI cases.

Of the 84 patients, 63 (75%) had underlying diseases and 13 (15·5%) patients had invasive diseases (bacteraemia). All the stool specimens from the 84 patients were negative for other enteric bacterial pathogens (*Salmonella, Shigella, Aeromonas, Plesiomonas*, *Campylobacter*, *Vibrio* spp.) The most common underlying disease of the 84 patients was haematological malignancy (40·5%, $n=34$), followed by endocrine diseases (22·6%, $n=19$). Fifty-one (60·7%) patients received antibiotic treatment (range 3 days to 3 months), 49 (58·3%) patients received proton pump inhibitors (PPIs), and 38 (45·2%) patients had a recent history of steroid usage. Fever (40·5%, $n=34$) was the most common clinical presentation, followed by abdominal discomfort (20·2%, $n=17$). Thirteen patients (15·5%) died during hospitalization. Further analysis revealed that patients who died were more likely than patients who survived to have received PPIs [odds ratio (OR) 9·3, 95% confidence interval (CI) 1·34–64·23, $P=0{\cdot}024$], to have invasive diseases (OR 7·7, 95% CI 1·27–46·35, $P=0{\cdot}026$), to have higher blood urea nitrogen levels ($24{\cdot}6\pm22{\cdot}3$ *vs.* $39{\cdot}42\pm29{\cdot}51$, $P=0{\cdot}04$), and to have higher alanine aminotransferase (ALT) levels ($41{\cdot}98\pm40{\cdot}81$ *vs.* $81{\cdot}29\pm61{\cdot}63$, $P=0{\cdot}03$).

Table 1 compares the clinical and demographic variables between the 50 patients with confirmed CDI and the 34 patients with probable CDI. The two groups of patients were similar with respect to age, sex, underlying diseases, drug exposure, and clinical symptoms. Only PPI usage differed significantly between the two groups of patients ($P=0{\cdot}02$). In addition, logistic regression analysis revealed that exposure to PPIs was a significant independent risk factor for confirmed CDI (OR 3·2, 95% CI 1·26–8·18, $P=0{\cdot}014$). Patients with confirmed CDI had higher levels of C-reactive protein ($P=0{\cdot}04$) (Table 2). The mortality rate did not differ between patients with confirmed CDI ($n=10$, 20%) and patients with probable CDI ($n=3$, 8·8%, $P=0{\cdot}35$). In addition, although patients who died were more likely than survivors to have been exposed to PPIs, there was no significant difference between the two groups of patients (90% *vs.* 65%, $P=0{\cdot}24$) (Table 1).

All of the 50 isolates from confirmed CDI patients contained both toxin A and toxin B genes (*tcdA*/*tcdB*). Seven (7/84, 8·3%) isolates carried both *tcdA*/*tcdB* and binary toxin genes (*cdtA*/*cdtB*) and six (6/84, 7·1%) of the seven isolates also possessed a deletion in the *tcdC* gene. Seven isolates had mutations in the *gyrA* gene and two isolates had changes in *gyrB* (Table 3). Only one isolate contained all toxin genes and mutations in both *gyrA* and *gyrB* genes (Table 3). None of the isolates belonged to the highly virulent PCR ribotypes 027 or 078.

There were no significant differences in clinical or laboratory findings between confirmed CDI patients who died ($n=10$) and those that survived ($n=40$) with the exception of haemoglobin level ($P=0{\cdot}01$). The mortality rate in confirmed CDI patients with isolates exhibiting deletion in the *tcdC* gene (2/6, 33·3%), those with isolates harbouring binary toxin genes (2/7, 28·6%), and those with isolates containing mutations in *gyrA* (2/7, 28·6%) and *gyrB* (1/2, 50%) was higher than the overall mortality rate in patients with confirmed CDI in our study; however, there were no significant differences. There was no significant difference ($P=0{\cdot}09$) in mortality between the patients who had isolates with ($n=6$) and without ($n=34$) the three genetic characteristics [*tcdA*/*tcdB*, binary toxin (*cdtA*/*cdtB*) genes and *tcdC* deletion] (Table 4). However, the differences were significant regarding serum sodium levels ($132{\cdot}3\pm4{\cdot}72$ *vs.* $137{\cdot}4\pm3{\cdot}98$, $P=0{\cdot}048$), and ALT ($10{\cdot}5\pm0{\cdot}71$ *vs.* $48{\cdot}3\pm49{\cdot}66$, $P=0{\cdot}001$) and aspartate aminotransferase (AST) ($15{\cdot}33\pm3{\cdot}79$ *vs.* $37{\cdot}93\pm31{\cdot}66$, $P=0{\cdot}002$) levels between these two groups (Table 4) in the univariate analyses. Further multiple logistic regression analysis did not show significant differences.

DISCUSSION

All virulent *C. difficile* strains contain five genes: *tcdR*, *tcdB*, *tcdE*, *tcdA*, and *tcdC*. The *tcdR* and *tcdC* genes are regulatory genes and the *tcdE* gene is a porin gene

Table 1. *Clinical characteristics of 84 patients with positive stool culture for* C. difficile *based on the presence* [*patients with confirmed* C. difficile *infection* (*CDI*)] *or absence* (*patients with probable CDI*) *of toxin genes of isolates*

Characteristic	Confirmed CDI (toxin gene-positive) ($n=50$)	Probable CDI (toxin gene-negative) ($n=34$)	*P* value
Age, years	59 ± 23	58 ± 20	0·88
Sex			0·38
Female	21 (42 %)	18 (52·9 %)	
Male	29 (58 %)	16 (47·1 %)	
Underlying disease			
Any	37 (74 %)	26 (76·5 %)	1·00
Solid tumour	9 (18 %)	7 (20·6 %)	0·78
Haematological malignancy	19 (38 %)	15 (44·1 %)	0·65
Endocrine diseases	13 (26 %)	6 (17·6 %)	0·43
Cardiovascular diseases	3 (6 %)	1 (2·9 %)	0·64
Genitourinary diseases	12 (24 %)	3 (8·8 %)	0·13
Operation	11 (22 %)	10 (29·4 %)	0·33
Antibiotic therapy			
Any	29 (58 %)	22 (64·7 %)	0·65
Penicillins	3 (6 %)	4 (11·8 %)	0·41
Cephalosporins			
Any	11 (22 %)	9 (26·5 %)	0·57
First generation	1 (2 %)	3 (8·8 %)	0·29
Second generation	0 (0 %)	0 (0 %)	
Third generation	4 (8 %)	4 (11·8 %)	0·69
Fourth generation	6 (12 %)	2 (5·9 %)	0·46
Fluoroquinolones	3 (6 %)	3 (8·8 %)	0·67
Carbapenems	7 (14 %)	4 (11·8 %)	1·00
Vancomycin	2 (4 %)	1 (2·9 %)	1·00
Metronidazole	1 (2 %)	1 (2·9 %)	1·00
Others	4 (8 %)	0 (0 %)	0·15
Steroid use	22 (44 %)	16 (47·1 %)	0·82
Anti-peptic ulcer drugs			
Proton pump inhibitor	35 (70 %)	14 (41·2 %)	**0·02**
H2 blocker	4 (8 %)	3 (8·8 %)	1·00
Symptoms			
Fever	20 (40 %)	14 (41·2 %)	0·90
Abdominal discomfort	11 (22 %)	6 (17·6 %)	0·84
Invasive diseases (bacteraemia)	6 (12 %)	7 (20·6 %)	0·36
Overall mortality	10 (20 %)	3 (8·8 %)	0·35

Bold values indicate significant difference ($P<0·05$).
Values given are mean ± standard deviation or *n* (%).

[16]. The *tcdA* and *tcdB* genes encode toxins A and B [16]. The *tcdC* gene is a negative regulator of the two major toxins. Defects in this gene may result in toxin over-expression [17, 18]. In addition to toxins A and B, highly virulent strains (ribotypes 027 and 078) tend to produce a third toxin (binary toxin). It is encoded by the genes *cdtA* and *cdtB* [19]. It is proposed that binary toxin has an additive effect with toxins A and B, thereby adding to the virulence of those strains [20].

Another characteristic of the highly virulent strains of *C. difficile* is their common resistance to fluoroquinolones [12]. Resistance to that class of antimicrobial agents has been shown to be associated with mutations in the *gyrA* and *gyrB* genes [12]. In addition, CDI due to highly virulent strains is associated with high morbidity and mortality rates. In this study, all the eight isolates exhibiting either *gyrA* ($n=6$), *gyrB* ($n=1$), or *gyrA* and *gyrB* ($n=1$) genes were all resistant to moxifloxacin (minimum inhibitory

Table 2. *Laboratory findings of 84 patients with positive stool culture for* C. difficile *based on the presence* [*patients with confirmed C. difficile infection* (*CDI*)] *or absence* (*patients with probable CDI*) *of toxin genes of isolates*

Finding	Confirmed CDI (toxin gene-positive) ($n=50$)	Probable CDI (toxin gene-negative) ($n=34$)	*P* value
White blood cell (cell/μl)	8660 ± 6652	6543 ± 4930	0·1
Haemoglobin (g/dl)	9·83 ± 1·67	9·81 ± 1·63	0·95
Neutrophil (%)	54·61 ± 35·28	62·90 ± 26·78	0·28
Platelet (cell/μl)	142660 ± 97511	145313 ± 99145	0·9
BUN (mg/dl)	30·51 ± 28·48	21·66 ± 13·97	0·07
Creatinine (mg/dl)	1·65 ± 1·77	1·09 ± 0·93	0·07
Sodium (mequiv/l)	135·01 ± 7·22	137·37 ± 3·98	0·08
Potassium (mequiv/l)	3·84 ± 0·7	3·78 ± 0·55	0·68
CRP (mg/dl)	7·61 ± 6·84	2·7 ± 2·54	**0·04**
Albumin (mg/dl)	3·50 ± 0·67	3·45 ± 0·67	0·83
Bilirubin, total (mg/dl)	1·48 ± 3·18	1·6 ± 2·37	0·89
Bilirubin, direct (mg/dl)	0·81 ± 1·16	0·82 ± 0·64	0·97
AST (IU/l)	51·05 ± 89·99	40·13 ± 31·63	0·52
ALT (IU/l)	46·94 ± 43·53	46·64 ± 47·94	0·98

ALT, Alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CRP, C-reactive protein.
Values given are mean ± standard deviation.
Bold values indicate significant difference ($P<0·05$).

Table 3. *Genotypic characteristics of 50* C. difficile *isolates from patients with confirmed* C. difficile *infection*

No. of isolates	*tcdA*/*tcdB*	*cdtA*/*cdtB*	*tcdC* deletion	Substitution(s) in *gyrA*	Substitution(s) in *gyrB*
36	+/+	–	–	–	–
5	+/+	+	+	–	–
1	+/+	+	–	–	–
6	+/+	–	–	Thr^{82} to Ile	–
1	+/+	–	–	–	Ser^{416} to Ala
1	+/+	+	+	Thr^{82} to Ile	Ser^{416} to Ala

concentration $\geqslant 4\,\mu$g/ml) [4]. However, we found no significant differences in mortality between patients with confirmed CDI and patients with probable CDI. A possible explanation for that finding is that our study populations were relatively small and that none of the patients had confirmed CDI due to highly virulent, NAP1 (ribotype 027)- or NAP8 (ribotype 078)-containing strains.

The risk factors for CDI include advanced patient age, hospitalization, gastrointestinal surgery, chemotherapy, and exposure to antimicrobial agents [9]. Another potential, although controversial, risk factor is the use of acid-suppressing medications, such as histamine-2 (H2) blockers and PPIs. In this study, we found that the rate of PPI usage was higher in patients with confirmed CDI. Some researchers have suggested that this association is the result of confounding factors such as underlying severity of illness and duration of hospital stay [2, 21, 22]. Interestingly, some studies have shown that patients with community-associated CDI are more likely to have received PPIs than patients that did not [23, 24], and that PPI usage is a risk factor for the development of CDI [25–27]. Paredes-Sabja *et al.* reported that reduction in gastric acid secretion might allow *C. difficile* to be ingested and that elevated pH levels (pH 6, determined to be optimal) might favour spore germination [28]. If acid suppression were in fact the true cause of *CDI* in patients receiving PPIs, then H2 blockers should also be associated with the development of CDI. In our study, however, H2 blockers were not associated with infections due to *C difficile*.

Table 4. *Clinical analysis of 40 patients who had isolates with and without the three genetic characteristics [tcdA/tcdB, binary toxin (cdtA/cdtB) genes and tcdC deletion]*

Characteristic	Patients with isolates with *tcdA*/*tcdB*, *cdtA*/*cdtB* genes and *tcdC* deletion		*P* value
	No ($n=34$)	Yes ($n=6$)	
Male sex	16 (47·1%)	2 (33·3%)	0·53
Any underlying disease	26 (76·5%)	5 (83·3%)	0·71
Death	3 (8·8%)	2 (33·3%)	0·09
Any antibiotic therapy	22 (64·7%)	2 (33·3%)	0·20
Operation	8 (26. 7%)	0 (0%)	0·71
Steroid use	16 (47·1%)	2(33·3%)	0·30
Anti-ulcer drugs			
Proton pump inhibitor	14 (41·2%)	2 (33·3%)	0·45
H2 blocker	3 (8·8%)	0 (0%)	1·00
Symptoms			
Fever	14 (41·2%)	2 (33·3%)	0·54
Abdominal discomfort	5 (14·7%)	1 (16. 7%)	0·59
Invasive diseases (bacteraemia)	7 (20·6%)	3 (50%)	1·00
Laboratory findings			
White blood cell (cell/μl)	6543 $\pm$ 4929·9	11073·3 $\pm$ 8324·1	0·07
Haemoglobin (g/dl)	9·81 $\pm$ 1·63	10·68 $\pm$ 1·64	0·24
Neutrophil (%)	62·89 $\pm$ 26·78	62·27 $\pm$ 47·86	0·97
Platelet (cell/μl)	145313 $\pm$ 99145	190667 $\pm$ 125371	0·33
BUN (mg/dl)	21·66 $\pm$ 13·97	54·18 $\pm$ 38·92	0·10
Creatinine (mg/dl)	1·09 $\pm$ 0·93	2·57 $\pm$ 2·76	0·25
Sodium (mequiv/l)	137·4 $\pm$ 3·98	132·3 $\pm$ 4·73	**0·049**
Potassium (mequiv/l)	3·78 $\pm$ 0·55	3·57 $\pm$ 0·40	0·53
CRP (mg/dl)	2·70 $\pm$ 2·54	12·00 $\pm$ 14·66	0·39
Albumin (mg/dl)	3·45 $\pm$ 0·67	3·2 $\pm$ 0·62	0·56
Bilirubin-total (mg/dl)	1·60 $\pm$ 2·37	0·78 $\pm$ 0·24	0·13
AST (IU/l)	37·93 $\pm$ 31·66	15·33 $\pm$ 3·79	**0·002**
ALT (IU/l)	48·30 $\pm$ 49·66	10·50 $\pm$ 0·71	**0·001**
Mortality	3 (8·8%)	2 (33·3%)	0·09
tcdA/*tcdB*, *cdtA*/*cdtB* genes and *tcdC* deletion			

ALT, Alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CRP, C-reactive protein.
Values given are mean $\pm$ standard deviation or *n* (%).
Bold values indicate significant difference ($P<0·05$).

A possible reason for that finding is that PPI therapy is a more potent inhibitor of gastric acid than H2 blockers, thereby increasing the intragastric pH. In a large pharmacoepidemiological cohort study, the occurrence of nosocomial CDI was 0·3% in patients receiving no acid suppression, 0·6% in those receiving H2 blockers, and 0·9–1·4% in patients receiving PPI therapy. A dose–response effect was found after adjustments for risk factors such as age, comorbid conditions, and antibiotic exposure. The odds ratio rose from 1·5 (H2 blocker), to 1·7 (daily PPI), to 2·4 (daily PPI) [29].

The mortality rate in patients with confirmed CDI in our study (20%) was similar to that of the crude mortality rate (23·5–24·8%) in other studies on patients with CDI [2, 5]. PPI usage by CDI patients contributed to a higher mortality rate than for those without PPI usage although the difference was not significant. PPI therapy is associated with a number of adverse reactions including increased susceptibility to bone fractures, infections (e.g. pneumonia, enteric infections, small intestinal bacterial overgrowth, spontaneous bacterial peritonitis), and altered gastric function (e.g. enteric malabsorption of vitamins and minerals, hypergastrinaemia-related neoplasia, impaired gastric emptying of solids) [18, 20]. All of those adverse reactions are associated with higher mortality for hospitalized patients. Mortality of hospitalized patients is also associated with the severity of underlying illness. In the present study, we found that

bacteraemia due to *C. difficile* was associated with a higher mortality rate.

Our study has several limitations. First, the real incidence of CDI at the hospitals was difficult to define because detection of toxins and toxin genes was not routinely performed for stool specimens of all patients with clinically suspected CDI. This might have resulted in bias in our findings. Second, we only screened toxin genes and PCR ribotyping of hypervirulent strains (ribotypes 027 and 078). Certain ribotypes with special characteristics might have been missed. Third, the PPI concentration and dosage were not investigated in this study. Although we found PPI usage to be a risk factor for the development of CDI, we were unable to evaluate whether there was a dose–response effect of PPIs.

In conclusion, we found no association between the presence of any toxin gene and the severity of CDI. Our data, however, demonstrated that PPI usage was associated with a higher risk of CDI. PPIs, therefore, should be administered with caution in patients with known risk factors for CDI, including patients of advanced age, those who have undergone gastrointestinal surgery, and patients that have been exposed to antimicrobial agents.

DECLARATION OF INTEREST

None.

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