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16S rRNA Gene As A Marker For Bacteremia In Patients With Acute Pancreatitis

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Abstract: Background: 16S ribosomal RNA (rRNA) gene polymerase chain reaction (PCR) is used for detection of bacterial pathogens in the serum of patients with acute pancreatitis (AP). Acute pancreatitis is a frequent cause of hospital admission. Infection and septic complications are the major factors contributing to the poor outcome in acute severe pancreatitis. PCR makes it possible to identify the presence of bacterial DNA in culture-negative samples from patients with suspected infection. This molecular approach allows non-culture rapid confirmation of pancreatitis and septicemia, which leads to improved disease surveillance and provides guidance on appropriate antibiotic usage and patient management. The aim of the present study is to detect bacterial DNA accurately by PCR targeting 16s rRNA as a common bacterial primer in serum of patients with acute pancreatitis in order to augment the standardized use of prophylactic antibiotics in such cases. **Methods:** twenty patients with acute pancreatitis aged 44.5±13.9 years, 6(30%) male and 14(70%) female patients were included. Serum amylase and CRP were measured. All patients were undergone genotyping for 16S rRNA gene by polymerase chain reaction to detect bacterial DNA in serum and conventional microbial blood cultures analyzed by BACTEC system. 60 samples were collected (3 for each case collected in the first 3 days after admission). **Results:** Bacterial DNA was detected in 13/60 (21.67%) samples from 8 patients by PCR analysis. Regarding these 8 patients, bacterial DNA was detected in 3 (37.5 %) cases on day 1; 7 (87.5 %) cases on day 2; and 3 (37.5 %) cases on day 3. All the 15 (100 %) cases who received antibiotics had negative blood culture with significant correlation ($p = 0.05$), and negative PCR1 with highly significant correlation ($P = 0.009$). 12of the15 cases (80 %) had negative PCR2 with significant correlation ($P = 0.03$) and 14 (93.3 %) cases had negative PCR3 results with no significant correlation ($P = 0.14$). Out of the 20 patients, only 2 (10 %) cases had positive growth in blood culture analyzed by BACTEC system. **Conclusion:** The PCR assay with universal primers targeting 16S rRNA genes is more sensitive in detecting the sepsis secondary to acute severe pancreatitis. Early antibiotic treatment reduced septic complications and mortality. But it remains a therapeutic challenge in the clinical management of A.P.

Key words: Polymerase chain reaction; Acute pancreatitis; Bacterial translocation; 16S rRNA.

INTRODUCTION

Acute pancreatitis (AP) is an acute inflammatory disease of the pancreatic gland that varies locally from mild pancreatic edema and inflammation to extensive infected pancreatic and peripancreatic necrosis, and systemically from a mild hypoxemia to multiple organ failure (OF) (Janet *et al*., 2012). Most acute pancreatitis episodes are mild and resolve itself without serious complications. However, approximately 20% of cases usually takes a severe clinical course and is associated with infected pancreatic necrosis and/or multiple organ dysfunctions; with a substantial increase of morbidity and mortality up to 40% (Petrov *et al*., 2010).

Infection and septic complications are the major factors contributing to the poor outcome in acute severe pancreatitis (Bettina *et al*., 2007). Infection of pancreatic and peripancreatic tissue in the course of acute pancreatitis occurs most frequently in patients with extensive pancreatic necrosis (Balzan *et al*., 2007). One third of patients with severe acute pancreatitis develop infectious complications that may explain up to 50% of deaths (Lozano *et al*., 2011). In the majority of cases infection is caused by bacterial translocation mostly gram-negative microbes from the gut lumen (Fritz *et al*., 2010).

Conventional microbiological blood culture methods are currently used widely, but may fail to yield positive results if the causative organism is fastidious in nature, cell dependent or has a fungal etiology even in patients with proven infected pancreatic tissue (Balthazar and Krinsky 2007). Molecular based diagnostic approaches are being increasingly employed, especially when a quick diagnosis is required. It has been shown that polymerase chain reaction (PCR) method is more sensitive than conventional blood cultures for detecting microbial products in blood (de Madaira *et al*., 2005). PCR using 16S rRNA-specific primers has identified bacterial DNA in blood; it is a highly conserved region of bacterial DNA, found in all Gram-positive and Gram-negative bacteria (Peng *et al*., 2010).

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The importance of PCR with respect to clinical application will depend on establishing a relationship between the presence or absence of clinical infection and the presence of bacterial DNA in the blood (Silvana *et al*., 2011). The PCR of bacterial DNA may provide information about the nature of inflammatory response in acute pancreatitis when traditional methods fail to detect bacteria, even in the presence of culture positive complications. It may also reveal more about the nature of susceptibility towards infective complications. In addition, sensitivity and accuracy of PCR could help target antibiotic therapy in the future (Kirstin *et al*., 2012).

The aim of the present study is to detect bacterial DNA accurately by PCR with the universal primers targeting 16s rRNA genes in serum of patients with acute pancreatitis in order to augment the standardized use of prophylactic antibiotics in such cases.

MATERIALS AND METHODS

Subjects:

This study was carried out on twenty newly diagnosed cases of acute pancreatitis patients admitted to emergency department of Ain Shams University Hospitals during the period of the clinical portion of the study from January to December 2011.The studied cases included 6 (30 %) male and 14 (70 %) female patients, with male to female ratio of 1: 2.3. Their age ranged from 23 to 72 years with mean age 44.40 ± 13.95 years.

All the patients were diagnosed as acute pancreatitis according to the following criteria: Severe epigastric pain radiating to the back, increase in serum amylase more than 3 times above the upper reference limit and abnormal pancreatic morphology on CT scan. All the studied cases were subjected to full history taking, clinical and general examinations. The severity of the disease was predicted by APACHE II score or Ranson score (Yeung *et al*., 2006). Written informed consent was obtained from each patient.

Samples:

Serum and blood samples were collected from each patient according to the following protocol: Blood from each patient obtained for routine haematological (CBC), coagulation (PT, PTT) and biochemical studies including serum amylase and C-reactive protein (CRP).

I. Ten ml of blood were obtained at day 2 after admission, and were inoculated at the bedside in blood culture bottles under aseptic conditions for analysis by BACTEC instrument.

II. Serum samples were obtained by centrifuging blood at 3500 rpm for 10 minutes, and collected at day 1, 2 and 3 for PCR analysis.

Methods:

a) Blood cultures for aerobic and anaerobic bacteria were processed using BACTEC instrument manufactured by BioMerieux.

b) PCR analysis of 16s rRNA gene was performed for all serum samples for accurate detection of bacteria in serum targeting the 16s rRNA as a common bacterial gene, through the following steps**:**

1. DNA extraction:

DNA was extracted with a QIAmp DNA Mini Kit supplied by (Qiagen, Hilden, Germany).

2. DNA amplification:

PCR reactions for the complete amplification of the 16s ribosomal RNA gene was carried out as follows:

a. Universal primers were:

- 5´-AGA GTT TGA TCA TGG CTC AG-3´ as forward (located at positions 8-27).
- 5´-GGT TAC CTT GTT ACG ACT T-3´ as reverse (positions 1509-1491).

b. The PCR reaction mixture consisted of 12.5 µl 2x PCR Master Mix {10xPCR buffer, 4 mM MgCl2, 0.5U Taq DNA Polymerase/µl and 0.4 mM deoxynucleotide triphosphate (dNTPs)}, 1 µl of each primer (25 pmol), 2.5 µl of genomic DNA and 8 µl nuclease free-H2O (Sigma Chemical Ltd) to complete up to the final volume of 25 µl.

c. Positive and negative controls were performed in duplicate in each assay to determine false results. DNA from Escherichia coli (E. coli) was added as positive control, and sterile water and PCR mixtures (without template) were used as negative controls.

d. PCR was carried out in a thermal cycler (Promega Corporation, USA) through the cycles protocol as follows:

- An initial preincubation at 95^{o} C for 4 minutes, then was followed by 35 cycles of 30 seconds denaturation at 95^{o} C, 30 seconds annealing at 61^{o} C and 30 seconds extension at 72^{o}C. There were final extension period at 72^{o}C for 10 minutes after completion of the cycling sequence.
- Total PCR reaction volume was filtered through QIAquick Spin Columns (QIAquick PCR Purification Kit; Qiagen) to remove primers and nucleotides.

3. Detection:

- Amplified products (6 µl) were detected by electrophoresis on 1.5% agarose gel stained with ethidium bromide and visualized by UV transillumination (Promega,USA).
- Bands of approximately 1500 bp (base pairs) were obtained, corresponding to the specific amplification of the 16s ribosomal RNA gene, as shown in figure(1):

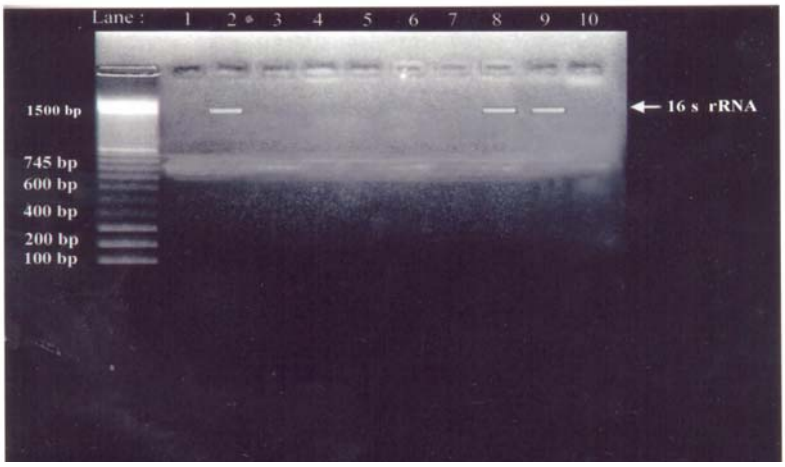

Fig. 1: Electrophoretic separation of PCR product for 16s rRNA (1500 bp):
Lane 1: negative control.
Lane 2: positive control of 16s rRNA.
Lanes 8 & 9: Positive results for 16s rRNA at days 3 & 1 of cases No. 11 & 12 respectively.

Statistical Analysis:

Results were analyzed using the SPSS (Statistical Package for the Social Science) software, version 15.0 (Chicago, IL, USA). Quantitative data were expressed as mean and standard deviation (SD) in case of parametric data and in the form of (median) range in case of non parametric data, while qualitative data was expressed in the form of number and percentage. Chi-square (x^2) test was used to test association between two qualitative variables. Quantitative data were compared using two sided student t-test, where significant p-value was ≤ 0.05.

Results:

Results of the present study are shown in tables (1-5) and figures (2&3). All the studied cases were observed for the development of complications during the period of follow-up.

Clinical data of the studied cases are shown in table 1. Out of the twenty studied cases, 8 (40 %) cases were presented with fever and all the 20 (100 %) cases presented with severe epigastric pain. 15 (75%) cases were receiving systemic prophylactic antibiotic (imipenem) to guard against pancreatic infection. Blood cultures showed bacterial growth in 2 (10%) out of the 20 cases.

Table 1: Statistical analysis of clinical data of acute pancreatitis patients:

Parameter	Frequency	Percentage
Male	6	30 %
Female	14	70 %
Gall stones	10	50 %
Post- ERCP	4	20 %
Fever	8	40 %
Epigastric pain	20	100 %
Recovered	17	85 %
Died	3	15 %
Receiving prophylactic antibiotic	15	75 %
Positive blood culture	2	10 %
Complication	—	—

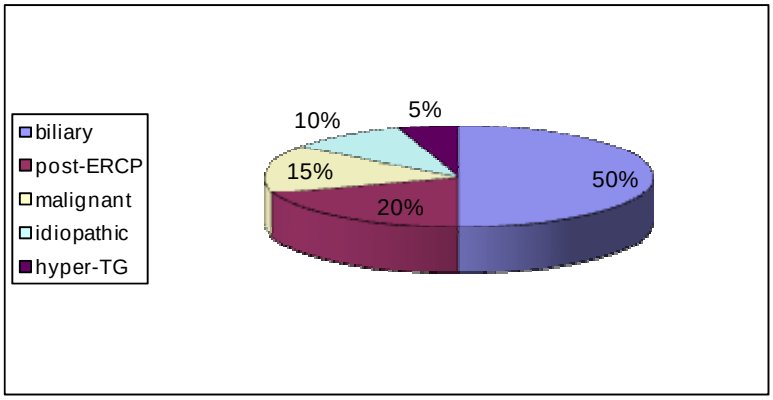

Fig. 2: Distribution of etiology among acute pancreatitis patients.

The etiology of A.P is shown in figure 2 as follows: (1) Gall stones (biliary) in 10 (50 %) cases, (2) post-ERCP in 4 (20 %) cases, (3) cancer pancreas in 3 (15 %) cases, (4) idiopathic in 2 (10 %) cases, (5) hyper-triglyceridemia in 1 (5 %) case in which the triglycerides level reached 1240 mg/ dl.

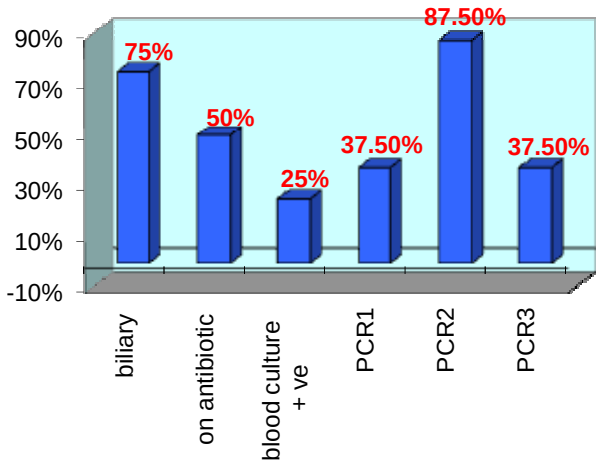

Fig. 3: Data of 16s rRNA positive acute pancreatitis patients.

Table (2): Characteristics of PCR positive patients:

Case No.	Aetiology	Fever	Proph. ATB	S. amylase	CRP	Blood culture	PCR			Outcome
							Day 1	Day 2	Day 3	
1	Biliary	-	+	800	163	Neg	Neg	Pos	Neg	R
5	Post-ERCP	-	+	1370	134	Neg	Neg	Pos	Pos	R
8	Biliary	+	-	980	94	Neg	Neg	Pos	Neg	R
11	Biliary	-	+	2012	34	Neg	Pos	Neg	Pos	R
12	Biliary	+	-	1026	112	Pos	Pos	Pos	Pos	R

13	Biliary	+	-	826	120	Neg	Neg	Pos	Neg	R
17	Biliary	-	+	1228	168	Neg	Neg	Pos	Neg	R
19	Idiopathic	+	-	2068	136	Pos	Pos	Pos	Neg	R

R = recovered Pos = positive Neg = negative
proph. ATB= prophylactic antibiotic

Bacterial DNA was detected in 13/60 samples (21.67%) from 8 patients by polymerase chain reaction (PCR) analysis (case No: 1, 5, 8, 11, 12, 13, 17 & 19). PCR was done on days 1, 2& 3 after admission. Out of the 8 patients, only 4 (50 %) cases were presented with fever and the aetiology was gall stones in 6 (75 %), post-ERCP in 1 (12.5%) case.

Only 4/8 (50 %) cases received prophylactic ATB (imipenem) on admission to emergency room, and the blood culture results showed positive growth in only 2 (25 %) cases of them (cases No: 12 & 19).

Regarding these 8 patients, bacterial DNA was detected in 3 (37.5 %) cases on day 1,7 (87.5 %) cases on day 2, and 3 (37.5 %) cases on day 3. only one case had positive PCR results in the 3 samples (case No: 12), and only one case of the 8 patients had negative PCR result on day 2 (case No: 11).

Level of serum amylase in these patients ranged from 800 to 2068 U/L and CRP levels ranged from 94 to 168 mg / dL and showed a trend towards higher levels in patients with bacterial DNA in the blood. All the 8 patients achieved recovery with no complications.

Table (3): Comparison between results of blood culture & PCR in acute pancreatitis patients:

Parameter		Blood Culture		P- Value	Significance
		- Ve	+ Ve		
PCR 1	Negative	16	1	0.284	N.S ($P > 0.05$)
	Positive	2	1		
PCR 2	Negative	13	0	0.111	N.S ($P > 0.05$)
	Positive	5	2		
PCR 3	Negative	17	0	0.016	S ($P < 0.05$)
	Positive	1	2		

Diphtheroids and E. coli were isolated in the positive blood cultures (cases No: 12 & 19 respectively). Regarding these 2 cases, one of them (50 %) showed positive PCR on day 1 and both cases (100 %) had positive PCR on day 2 with no significant correlation ($P > 0.05$). Bacterial DNA was isolated from both cases on day 3 with significant correlation ($P < 0.05$).

Table 4: Laboratory Findings of patients received prophylactic antibiotic:

Parameter		Proph. ATB		P-value	Significance
		- Ve	+ Ve		
Bl. Culture	- Ve	3	15	0.05	S
	+ Ve	2	0		
PCR 1	- Ve	2	15	0.009	H.S ($P < 0.01$)
	+ Ve	3	0		
PCR 2	- Ve	1	12	0.031	S ($P < 0.05$)
	+ Ve	4	3		
PCR 3	- Ve	3	14	0.14	N.S ($P < 0.05$)
	+ Ve	2	1		

+Ve = positive - Ve = negative
proph.ATB= prophylactic antibiotic

All the 15 (100 %) cases who received antibiotics had negative blood culture with significant correlation ($p = 0.05$), and negative PCR1 with highly significant correlation ($P = 0.009$). 12 of the 15 cases (80 %) had negative PCR2 with significant correlation ($P = 0.03$) and 14 (93.3 %) cases had negative PCR3 results with no significant correlation ($P = 0.14$).

Out of the 5 patients who did not receive antibiotic, 2 (40%) cases had positive blood culture, 3 (60 %) cases had positive PCR1, and 4 (80 %) cases had positive PCR2 and 2 (40 %) cases showed positive PCR3 results.

Table 5: Results of patient's outcome in relation to laboratory and clinical data

		Outcome		P-value	Significance
		Recovered	Died		
Blood Culture	- Ve	15	3	0.710	N.S (P > 0.05)
	+ Ve	2	0		
PCR 1	- Ve	14	3	0.596	N.S (P > 0.05)
	+ Ve	3	0		
PCR 2	- Ve	10	3	0.251	N.S (P > 0.05)
	+ Ve	7	0		
PCR 3	- Ve	14	3	0.596	N.S (P > 0.05)
	+ Ve	3	0		
On Antibiotic	- Ve	5	0	0.399	N.S (P > 0.05)
	+ Ve	12	3		

Follow-up was done for the 20 studied cases; 17 (85 %) cases achieved complete recovery, 2 of them had positive blood culture, 3 were positive for PCR1, 7 were positive for PCR 2, 3 positive for PCR 3, and 12 cases were receiving prophylactic antibiotic.

All the 3 cases that showed complications and died had negative in blood culture and PCR results. The etiology of A.P in these 3 patients was malignancy, they died mostly due to metastatic complications, and all of them were receiving prophylactic antibiotic.

Blood culture and PCR results showed non-significant statistical difference between the group that achieved recovery and the group that showed complications and died ($P > 0.05$).

Discussion:

Bacterial infections are common complications in patients with acute pancreatitis (A.P), and translocation of bacteria from the intestine is probably the first step in the pathogenesis of these infections (Silvana *et al*., 2011). As blood cultures in afebrile patients are usually negative, more sensitive techniques for the fast and accurate detection of bacterial infection are critical for early diagnosis, prevention and treatment of bacterial translocation in severe AP (Peng *et al*., 2010).

The aim of the present study is to detect bacterial DNA accurately by PCR targeting 16s rRNA as a common bacterial primer in serum of patients with acute pancreatitis in order to augment the standardized use of prophylactic antibiotics in such cases.

In our study, we performed blood cultures and PCR analysis as a more sensitive test to detect 16s rRNA as a common bacterial primer. Blood cultures showed bacterial growth in 2 (10%) out of 20 cases. The isolated organisms were Diphtheroids in case No: 12 and E. coli in case No: 19. PCR was performed on 60 samples (3 for each case collected in the first 3 days after admission) and bacterial DNA was detected in 13/60 (21.67%) samples from 8 patients. Out of the 8 patients only 2 cases had positive blood cultures (cases No: 12 & 19). Case No. 12 had positive PCR analysis in all the 3 samples, while case No. 19 had positive PCR in the first 2 days only. The 13 samples positive for bacterial DNA were as follows: 3 (23.1 %) samples in day 1; 7 (53.8 %) samples in day 2; and 3 (23.1 %) samples in day 3. This is in accordance with the results of Zhang *et al*., (2007) and Aydin *et al*., (2009).

Zhang *et al*. (2007) performed PCR on blood samples from patients with acute necrotizing pancreatitis. They reported a PCR detection of bacterial DNA in 8 (33.35%) out of 22 tested samples and all positive samples were found to contain E. coli, all the samples were taken during periods of likely sepsis. Aydin *et al*., (2009) studied six patients, and blood samples of their patients were collected at days 1, 3 and 5 after admission, for both blood culture and PCR analysis. PCR detected the presence of bacterial DNA in 4 (23.6 %) out of the 17 samples, and all blood cultures showed no growth. In their study, the only patient who had complications and sepsis was PCR – positive for bacterial DNA in 2 out of 3 samples.

In contrast to our results, Ammori *et al*. (2003) failed to detect bacterial DNA by performing PCR on blood samples from 26 patients with A.P. This conflicting result may be due to different methodological problems, such as the level of sensitivity of the PCR or the search for bacterial DNA in whole blood, as it has been reported that both haemoglobin and lactoferrin may inhibit PCR.

Although the reasons why pancreatic necrosis becomes infected remain debatable, experimental evidence supports the hypothesis of bacterial translocation (BT) as the main pathogenic mechanism depending on that most pancreatic infections are caused by Gram negative bacteria of intestinal origin (Madaria *et al*., 2005). However, the underlying pathophysiology of bacterial translocation is poorly understood. Different hypotheses concerning the spread of enteric bacteria have been discussed. Although some authors suggest that bacteria cross the intestinal barrier and break into blood vessels (hematogenous dissemination), others suggest a lymphatic spread of intestinal bacteria (Van Minnen *et al*., 2007). In an experimental study, Fritz *et al*., (2010) concluded that bacterial translocation occurs mainly via mesenteric lymph nodes and not via hematogenous dissemination or a transperitoneal pathway and that bacterial translocation derives from the small bowel rather than from the colon.

In our study, the main isolate was E. coli. 2 (10%) patients had positive blood culture, E. coli was isolated from one case and Diphtheroids were isolated from the other one, and the latter may be explained as skin contamination of blood culture. This is in agreement with the results of Zhang *et al*. (2009), Ammori *et al*. (2003) and Madaria *et al*. (2005).

Early antibiotic treatment remains a therapeutic challenge in the clinical management of A.P and several papers have been published in this field (Pezzilli, 2006).

In our study, 15/20 cases (75%) received prophylactic antibiotics during the course of A.P. Antibiotic treatment was started 1-2 days after hospitalization, and the antibiotic used was imipenem. During follow-up 3/20 cases died mostly due to metastatic complications. The etiology was malignancy (cancer pancreas) in the latter 3 cases, they were receiving prophylactic antibiotics and no bacterial DNA was detected in their blood. No pancreatic or extrapancreatic infective complications were detected in any of the 20 studied cases; although bacterial DNA was detected by PCR in serum of 4/15 cases of the group that received antibiotic, with no significant difference between the group receiving antibiotic and the group not receiving antibiotic. This is in accordance with the results of Isenmann *et al*. (2004), Dellinger *et al*. (2007), Yang *et al*. (2009), and Xue *et al*. (2009).

Isenmann *et al*. (2004) demonstrated no advantage of early antibiotic prophylaxis (ciprofloxacin and metronidazole) in preventing infection in pancreatic necrosis in 114 studied patients. The authors reported a significant increase of infection by ciprofloxacin resistant organisms in the antibiotic group.

In contrast, Nord back *et al*., (2001) and Manes *et al*., (2006) reported in their studies that antibiotic prophylaxis with imipenem- cilastatin therapy appeared to reduce pancreatic infection in patients with severe A.P patients. In 2008, Fritz *et al*. proved in their study that both prophylactic and delayed antibiotic treatment on-demand reduced septic complications in a standardised setting of experimental necrotising pancreatitis. However, pancreatic superinfection, bacteraemia and mortality rates were reduced significantly by early treatment. Therapeutic administration of meropenem on-demand reduced bacteraemia to 50% and mortality to 27.3%. However, prophylactic antibiotic treatment significantly reduced bacteraemia to 25.0% ($p = 0.04$) and pancreatic superinfection as well as mortality to 0% ($p < 0.001$ and $p = 0.05$, respectively) compared with controls. Thus, the study demonstrates that prophylactic antibiotic treatment is superior to antibiotic treatment on-demand Fritz *et al*., (2010).

It is recommended to routinely use early antibiotic prophylaxis for cases with suspected complications or liable to infection of pancreatic necrosis in acute pancreatitis:

1- Use meropenem or imipenem / Cilastatin instead of Ampicillin, as the latter does not reach the pancreas.

2- Stop the use of prophylactic antibiotics in mild forms of pancreatitis with zero risk of infection.

3- Perform wide scale study on all groups of pancreatitis to reach a final conclusion for the debate of antibiotic use in acute pancreatitis.

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