

# Clinical Evaluation of Bacterial DNA Using an Improved Droplet Digital PCR for Spontaneous Bacterial Peritonitis Diagnosis

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## Research Article

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## **Abstract**

## **Objective**

Bacterial DNA (bactDNA) detection has been studied on ascitic fluid. However, there is insufficient data to support its use in clinical practice. We improved a novel droplet digital PCR (ddPCR) method and enhanced its diagnostic efficiency for spontaneous bacterial peritonitis (SBP).

## **Method**

A total of 250 patients were included in this retrospective study. Extra cell-free DNA was depleted using Benzonase before pathogen DNA extraction to obtain viable bacterial DNA. The threshold value of bactDNA quantitation and its diagnostic performance were established based on ascites-polymorphonuclear (PMN) and clinical manifestation. The bactDNA quantification analysis were detailedly performed on patients who were symptomatic and had a PMN < 250 cells/mm<sup>3</sup>.

## **Results**

This study enrolled 191 patients with liver cirrhosis and ascites. After the removal of free DNA, bactDNA detected by ddPCR were generally decreased(1.75 vs 1.5 copies/μl, P<0.001), while the area under the curve for diagnosing SBP was increased, which 0.98 for total, 0.91 for gram-positive, 0.95 for gram-negative bactDNA. Compared with traditional culture and PMN count, results based on composite diagnostic standard showed that the sensitivity of ddPCR testing was 80.5% for total, 72% for Gram-positive, and 93.9% for Gram-negative bactDNA while the specificity was 95.3%, 93.9%, and 89.3%, respectively. In patients with PMN <250 cells/mm<sup>3</sup>, the bactDNA quantitation of 13 patients who were symptomatic was significantly higher than those asymptomatic(2.7 vs 1.7 copies/μl, P<0.001).

## **Conclusion**

BactDNA quantitation in ascites by ddPCR is a promising approach to improve the diagnostic accuracy of SBP, especially for symptomatic patients with PMN < 250 cells/mm<sup>3</sup>.

## **Introduction**

Spontaneous bacterial peritonitis (SBP) is an infectious disease caused by pathogenic microorganisms that invade the abdominal cavity and cause obvious damage<sup>[1]</sup>. In people with end-stage liver diseases, the incidence rate of SBP can even reach to 40% - 70%<sup>[2]</sup>. Currently, SBP defined as an ascites-polymorphonuclear count (PMN) greater than 250 cells/mm<sup>3</sup><sup>[1][2][3]</sup>. However, 60% - 80% of patients with PMN count < 250 cells/mm<sup>3</sup> had signs and symptoms<sup>[4][5]</sup>; of those, 38% developed SBP<sup>[6]</sup>. Additionally,

antibiotics used empirically only based on clinical symptoms or signs and PMN can lead to the excessive application of antibiotics and the occurrence of multidrug resistant organisms<sup>[7][8]</sup>. Current traditional culture for SBP have insufficient sensitivity to detect samples with bacteria<sup>[1]</sup>, especially those with low bacteria loads. Therefore, it is urgent to introduce more accurate and rapid etiological diagnosis methods.

Recently, ascites-bacterial DNA (bactDNA) are expected to become a rapid and suitable marker to replace the general bacterial culture of ascites to find infectious pathogens<sup>[2][9]</sup>. Droplet digital polymerase chain reaction (ddPCR) is a novel absolute quantitative molecular detection technology developed in recent years, which has high sensitivity, simple and quick operation without relying on the standard curve<sup>[10][11]</sup>. The ddPCR technology produces about 20,000 droplets and enriches target DNA by reducing competition with high-copy templates. After PCR amplification in each droplet, the concentration of target DNA can be determined based on Poisson algorithm according to fluorescence positive and negative droplet. Studies have suggested that the ddPCR technology was able to detect very low amounts of pathogen DNA within 4 hours and had been applied in the diagnosis of bloodstream and tuberculosis infections<sup>[11][12]</sup>. This provides a direction for the application of ddPCR in clinical diagnosis of bacterial infection.

Nevertheless, some previous studies<sup>[13][14][15]</sup> had shown that there was no strong correlation between the presence of bactDNA in ascites and the occurrence of SBP, so that the diagnosis of SBP by bactDNA has not been readily applied in clinical practice. The possible reasons are as follows: 1) the numbers of bacteria plays a very important role in the development of SBP, not just the presence of bactDNA<sup>[16]</sup>; 2) the large volume of ascites that often occurs in patients with cirrhosis may dilute bacterial products<sup>[17]</sup>. Whether dilution is an important influencing factor; 3) accumulated evidence indicates<sup>[18]</sup> that bacteria may translocate either as a viable or non-viable form, and the migration of nucleic acid DNA may potentially obscure any correlation between microbes and clinical parameters.

Therefore, the purpose of our study was to assess the quantitation of viable bactDNA in ascites for the diagnostic accuracy of SBP, using an optimized method of ddPCR.

## Methods

### Study design and population

This study was approved by the ethical committee of Beijing YouAn Hospital, Capital Medical University and was done according to good clinical practice. A total of 250 patients with liver cirrhosis and ascites from the Liver Disease Center, Beijing YouAn Hospital, Capital Medical University between March 1, 2020 and December 31 , 2020 were retrospectively included in the study. The samples of cancerous ascites, secondary peritonitis, intake of antibiotics in the preceding 2 weeks and incomplete clinical data were excluded. The study design was composed of a laboratory study and a clinical study (Fig.1). Clinical data of the patients enrolled in the study was obtained by medical record review and was analyzed to determine the likelihood of an infection. The diagnosis of SBP is based on PMN (2021 practice guideline)<sup>[3]</sup> and a clinical composite diagnosis (2017 Chinese guidelines)<sup>[19]</sup>that incorporated 1) clinical signs or

symptoms; 2) laboratory test abnormalities; 3) adjudication independently by an infectious disease specialist (C.L.H) and two liver disease experts (Y.H and W.H). Patients were firstly classified as spontaneous bacterial peritonitis (SBP: PMN > 250/mm<sup>3</sup> with positive ascites culture), culture negative neutrocytic ascites (CNNA: PMN > 250/mm<sup>3</sup> with negative ascites culture), monomicrobial non-neutrocytic bacterascites (MNB: PMN < 250/mm<sup>3</sup> with positive ascites culture) and the absent of ascitic fluid infection (no-AFI)<sup>[1][3]</sup>. Patients with overt clinical symptoms and signs but with a PMN count < 250/mm<sup>3</sup> were further classified as suspected SBP, bacterascites and no-AFI (Fig.1b)<sup>[20][21][22]</sup>. The inclusion and exclusion criteria are detailed in the Supplementary Data.

All ascites samples were obtained by paracentesis under sterile conditions according to routine procedures upon admission. To evaluate and eliminate the influence of ascites volume on bacterial quantity, we adopted the study of ultrasonic three-point method from Hirooka M et al.<sup>[23]</sup> (Fig.1b), which calculated the total amount of ascites: total amount (ml) = (1100/3) × (A + B + 0.5 × C). In this way, the absolute amount of bacteria could be determined by total ascitic volume ([ml] × bacterial ddPCR [copies/μl]).

## ddPCR methods

**Primes and probes.** The 20 bacteria sequences of primers and probes were adopted and revised from the previous article and synthesized by Sangon Biotech (Shanghai) Co, Ltd.<sup>[12]</sup>. The reaction conditions were optimized and screened according to the requirements of ddPCR. Then, the performance of primers and probes was verified, which included sensitivity, specificity, linearity, and repeatability.

**Sample processing.** To deplete the extracellular DNA from the ascites samples, 1 ml samples were centrifuged at 4°C with 13000r for 10 minutes. After discarded 760 μl supernatant, we added 40.5 μl mixture of buffer and benzonase endonuclease and incubated this mixture at 37 °C for 15 minutes. Next, we added 20 μl of protease K and incubated it at 56°C for 20 minutes to inactivate the Benzonase.

**DNA extraction.** 400 μl 2 × DNA / RNA shield (zymo R1200-125) was added into each of the pre-treatment samples. After mixing, the samples were homogenized using a program of 4 °C 60Hz for 120 seconds, stopped for 20 seconds, repeated for 4 times in Tissuelyser (Servicebio, Wuhan, China), and then centrifuged at 10000r for 2 minutes. We then individually added 400 μl DNA/RNA analysis buffer, prep buffer and wash buffer for repeated DNA washing. Finally, 50 μl DNase/RNase-free water was added to collect target DNA and stored at - 80°C until tested.

**Droplet preparation and detection.** ddPCR was performed with the TargetingOne Digital PCR System (TargetingOne, Beijing, China). The master mix for ddPCR included 1× ddPCR supermix for probes, 400 nmol/L of forward and reverse primers, and 200 nmol/L GRAM+/GRAM- probes; 1 μl sample of DNA and DNase/RNase Free water were mixed together, and the final volume was 30 μl for each well. The droplet was generated according to the manufacturers' protocols.

PCR amplification was performed with the following conditions: 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 10 minutes; annealing and extension was at 60°C for 1 minute. The strip tubes were stored at 4°C until the droplets were analyzed with a TargetingOne chip reader and TargetingOne ddPCR Analyzer 1.0. The threshold between positive and negative droplet populations was manually set using per-plate positive and no-template controls as a guide.

### **Statistical analysis**

The data is presented as a mean  $\pm$  standard deviation or median with a range. We used the Wilcoxon-Mann Whitney tests for between-group comparisons or Kruskal-Wallis test with post-hoc tests for continuous data. The continuous variables were dichotomized according to the maximum approximate index in the receiver operating characteristic (ROC). The statistical analyses were performed using SPSS software, versions 24 (IBM, Armonk, NY), and Prism 8 (GraphPad, La Jolla, CA). In the two-sided test, a P < 0.05 was considered statistically significant.

## **Results**

### **Patient Characteristics**

Two hundred and fifty patients were enrolled in this study. The ascites of 7 patients were malignant ascites. Secondary peritonitis occurred in 6 patients, and 46 patients without complete data were excluded. One hundred and ninety one patients were enrolled, and their ascites samples were collected. Among them, 155 were male (81.2%), with an average age of  $58.1 \pm 9.1$  years. Liver cirrhosis was caused by alcoholic hepatitis in 73 cases (38.2%), viral hepatitis B in 71 cases (37.2%), viral hepatitis C in 7 cases (3.7%), and other causes in 40 cases (21.0%). There were 41 suspected SBP cases (21.5%), 18 bacterascites cases (9.4%), and 132 no-AFI (69.1%). The demographic data are listed in Table 1.

Table 1  
Clinical characteristics of enrolled patients.

	<b>SBP</b> <b>(n=41)</b>	<b>Bacterascites</b> <b>(n=18)</b>	<b>No-AFI</b> <b>(n=132)</b>	<b>P*</b>
Age (years, mean ± S.D.)	58.6 ± 8.3	59.4 ± 10.8	57.8 ± 9.2	0.766
Gender (Male/Female)	37/4	14/4	104/28	0.298
Etiology				
Alcohol	17 (41.5)	9 (50.0)	47 (35.6)	0.934
HBV	15 (37.5)	6 (33.3)	50 (37.9)	
HCV	1 (2.5)	0(0)	6 (4.5)	
HBV plus alcohol	4 (9.8)	1 (5.6)	9 (6.8)	
Others	4 (9.8)	2 (11.1)	20 (15.2)	
Complications				
Ascites 2/3	24/17	10/8	80/52	<0.001
Gastrointestinal bleeding	3 (7.5)	2 (11.1)	12 (9.0)	<0.001
Hepatic encephalopathy	4 (10.0)	2 (11.1)	17 (12.9)	<0.001
HRS	5 (12.5)	1 (5.6)	22 (16.7)	<0.001
Laboratory parameters				
ALT (IU/L), median (IQR)	18.2 (8.1,28.3)	17.4 (10.1,24.3)	19.5 (11.7,31.6)	0.297
AST(IU/L), median (IQR)	36.5 (18.7,63.7)	24.2 (15.8,49.8)	39.6 (24.9,61.6)	0.147
albumin (g/dL), mean ± SD	30.2 ± 4.7	29.3 ± 4.4	29.7 ± 4.1	0.741
Total bilirubin (μmol/L), median (IQR)	54.8 (15.4,168.5)	34.4 (21.3,46.9)	52.3 (26.6,99.8)	0.282
PTA, mean ± SD	65.7 ± 30.9	59.2 ± 16.8	58.7 ± 18.7	0.231

SD, standard deviation; IQR, interquartile range; HRS, hepatorenal syndrome; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PTA, prothrombin activity; CTP, child-turcotte-pugh score; MELD, model for end-stage liver disease; PMN, polymorphonuclear neutrophils; WBC, white blood cell. AFI, ascitic fluid infection.

\*P value from Kruskal-Wallis test for continuous variables or Fisher's exact test for discrete variables comparing patients with SBP to patients with bacterascites and No-AFI.

\*P<0.05

	SBP (n=41)	Bacterascites (n=18)	No-AFI (n=132)	P ¶
Serum creatinine ( $\mu\text{mol/L}$ ), median (IQR)	91.2 (63.8,179.5)	84.4 (64.8,134.0)	81.7 (59.5,120.3)	0.422
Platelets ( $10^9/\text{L}$ ), median (IQR)	91.5 (63.4,141.3)	53.2 (26.2,72.7)	79.5 (45.5,114.8)	0.008
Ascites WBC count ( $\times 10^6/\text{L}$ ), median (IQR)	1693 (765,3620)*	220 (92,322)	200 (130,620)	<0.01
Ascites PMN count ( $\times 10^6/\text{L}$ ), median (IQR)	1437 (1123,3587)	40 (8,111)	54 (28,94)	<0.01
Scores				
CTP	$10.1 \pm 1.8$	$9.6 \pm 1.4$	$10.0 \pm 1.6$	0.688
MELD	$12.9 \pm 9.8$	$9.6 \pm 5.2$	$11.9 \pm 7.9$	0.383
SD, standard deviation; IQR, interquartile range; HRS, hepatorenal syndrome; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PTA, prothrombin activity; CTP, child-turcotte-pugh score; MELD, model for end-stage liver disease; PMN, polymorphonuclear neutrophils; WBC, white blood cell. AFI, ascitic fluid infection.				
¶P value from Kruskal-Wallis test for continuous variables or Fisher's exact test for discrete variables comparing patients with SBP to patients with bacterascites and No-AFI.				
*P<0.05				

## Analysis And Valuation Of Ddpcr Method

Results showed that the clustering effect of ddPCR was best when the primer probe concentration was 400/200 nmol/L and the annealing temperature was 60 °C(Supplementary Table S1, Fig. 1). Representative Gram-positive and Gram-negative bacteria were selected for a specific probe test, which indicated that the probe could clearly distinguish Gram-positive or Gram-negative bacteria (Fig. 2a, b). To determine whether the probe had cross interference in distinguishing Gram-positive and Gram-negative bacteria, we mixed *E. faecium* and *E. coli* in different concentrations (100:1, 1:100, 1:1) and tested them. The results showed that the corresponding quantitative level of bactDNA were 4.3: 2.1 log copies/ $\mu\text{l}$ , 2.0 : 4.0 log copies/ $\mu\text{l}$ , and 2.2 : 2.3 log copies/ $\mu\text{l}$ , indicating that reaction systems with different concentrations could be accurately classified and quantified (Fig. 2c, d, e).

To determine the ddPCR detection limit, linearity, and repeatability, 9 kinds of bacteria were spiked in a mixed system and subsequently detected by ddPCR. Serial dilutions of the above bacteria at known concentrations showed a good linearity ( $R^2 = 0.97\text{--}0.99$ ; Supplementary Fig. 2 ), with three replicates at each dilution (1 - 5 log copies); the ddPCR detection limit was approximately 20 - 45 copies/ $\mu\text{l}$  for

bacterial strains. Compared with traditional qPCR methods, the ddPCR showed better linearity and lower detection limits (Supplementary Fig. 3).

## Clinical Evaluation Of An Improved Quantitation Of Ddpcr Method

Benzonase endonuclease digestion was performed on the samples before detection, so that all detected DNA came from live bacteria. We selected randomly 54 cirrhotic ascites samples (13 SBP, 15 bacterascites, 26 no-AFI samples). Results showed that compared to the extraction method of None-dependent assay, Benzonase-dependent assay had a significant difference ( $P < 0.001$ ) and even lower quantitation of bactDNA (1.75 vs 1.5 copies/ $\mu$ l, Fig. 3a, b). And then we found that this difference between the two methods of extraction was mainly the extraction of gram-negative bacteria from bacterascites and no-AFI (Fig. 3c), which were conducive to distinguish the interference of cell-free DNA fragments and the real bacterial infection with low loads. After the depletion of DNA fragments, the area under the ROC curve were as follows: bactDNA 0.98 (95%CI, 0.94-1.00), Gram-positive bactDNA 0.91 (95%CI, 0.84-0.99), Gram-negative bactDNA 0.95 (95%CI, 0.88-1.00; Fig. 3d), which indicated that DNA treatment with benzonase may have a more diagnostic value.

The clinical conditions of cirrhotic patients with ascites are complex and varied, and the change of ascites volume may also affect the judgment of the absolute numbers of bacteria. We used the ultrasonic three-point method to evaluate and eliminate the influence of ascites volume and found that there was no significant variation in bactDNA quantitation between the copies per 1  $\mu$ l ascites and the total copies of bactDNA combined with ascites volume ( $P > 0.53$ , Fig. 3e). Compared with bactDNA quantification in different ascites samples, the quantitation of bactDNA in the ascites of SBP patients (total 2.8, gram-positive 1.9, gram-negative 2.5 log copies/ $\mu$ l) were significantly higher than that of bacterascites (1.7, 1.3, 1.5 log copies/ $\mu$ l) and no-AFI (2.0, 1.4, 1.8 log copies/ $\mu$ l,  $P < 0.001$ , Fig. 3e, Supplementary Table S2), while the bactDNA of bacterascites and no-infectious ascites had no significant differences.

### The enhanced sensitivity and specificity of bactDNA facilitated SBP diagnosis

Subsequently, we plotted ROC curves at varying bactDNA quantification corresponding to SBP analysis. Results from 191 samples showed that the cutoff value of bactDNA quantification was 103.2 copies/ $\mu$ l comparing to the diagnosis of SBP. For patients infected with Gram-positive bacteria and Gram-negative bacteria, the cutoff values were 37.3 and 68.6 copies/ $\mu$ l, respectively (Fig. 4a).

At the optimal Yueden's index derived from the ROC curve, the sensitivity and specificity of total bactDNA quantitation comparing to the composite clinical standard were 80.5% (95%CI, 67.8%-93.2%) and 95.3% (95%CI, 91.1%-98.3%), the positive percentage agreement (PPA) and negative percentage agreement (NPA) were 82.5% and 94.7%, respectively. And the diagnostic test revealed sensitivity 72% (95%CI, 53.1%-90.9%), specificity 93.9% (95%CI, 90.3%-97.6%), PPA 64.3% and NPA 95.7% of SBP caused by Gram-positive bacteria, and sensitivity 91.3% (95%CI, 78.8%-100%), specificity 89.3% (95%CI,

84.6%-94.0%), PPA 53.8% and NPA 98.6% of SBP caused by Gram-negative bacteria (Fig. 4b). Additionally, the agreement of bactDNA positive results with culture, PMN, composite diagnostic standard positive results was 56.8% (95%CI, 40.0%-73.5%), 71.4% (95%CI, 53.6%-89.3%), and 80.5% (95%CI, 67.8%-93.2%). The negative results agreement among them was 88.3% (95%CI, 83.2%-93.4%), 87.7% (95%CI, 82.6%-92.8%), and 95.3% (95%CI, 91.1%-98.3%), respectively (Table 2). Therefore, as a novel tool, bactDNA based on ddPCR greatly improves microbial diagnosis in SBP.

Table 2

Positive and Negative Agreement of quantitation of bactDNA of SBP versus culture, PMN, and composite diagnostic standard

Outcome (n=191)	Total bactDNA-positive	Total bactDNA-negative	Agreement(%)
Traditional culture-positive (n=37)	21	16	56.8
Traditional culture-negative (n=154)	18	136	88.3
PMN $\geq 250/\text{mm}^3$ (n=28)	20	8	71.4
PMN $< 250/\text{mm}^3$ (n=163)	20	143	87.7
Composite diagnostic standard-SBP (n=41)	33	8	80.5
Composite diagnostic standard-no-SBP (150)	7	143	95.3

\*PMN polymorphonuclear; SBP spontaneous bacterial peritonitis.

### BactDNA quantitation in patients with symptoms and PMN count $< 250/\text{mm}^3$ can sensitively distinguish patients with suspicious infection

The data showed that 163 out of 191 samples had PMN less than 250 cells/mm<sup>3</sup>, of which 13 patients were consulted by 2 infectious physicians and 1 hepatologist to consider the diagnosis of SBP and were treated with empirical antibiotics (see Supplementary Table S3). After treatment, the patients' symptoms improved and was clinically confirmed as SBP. In 13 SBP patients with a PMN < 250 cells/mm<sup>3</sup>, the quantitation of bactDNA in ascites were significantly higher than those in no-SBP patients (2.7, 2.2, 2.1 log copies/μl vs 1.7, 1.1, 1.5 log copies/μl, P < 0.001, Fig. 4c, Supplementary Table S4). Notably, we found that 7 patients with bacterascites were all culture-positive (Fig. 4d), including *Enterococcus faecalis*, *Enterococcus faecium*, *Staphylococcus haemolyticus*, *Corynebacterium*, which were consistent with previous study in bacterascites<sup>[6]</sup>. According to the counts of PMN from both culture and ddPCR, those of gram-positive bacteria were lower than gram-negative bacteria (Fig. 4e). On the view of our data, the cutoff value of PMN count was 192 cells/mm<sup>3</sup> with 73.2% (95%CI, 59.4%-87.2%) sensitivity and 98.6% (95%CI, 97.3%-100%) specificity compared to composite diagnostic standard. Therefore, we hypothesized

that a PMN threshold of  $250/\text{mm}^3$  was too high for the diagnosis of SBP, especially for Gram-positive infections.

## Discussion

In this study, we described a rapid diagnostic method for detection of bactDNA with low loads based on ddPCR to evaluate the bactDNA quantification of abdominal infection in a large series of liver cirrhosis patients with ascites. To our knowledge, this is the first study reporting detection of vital bacteria using benzonase to improve the diagnosis of SBP. Particularly, for patients with symptom and PMN count  $<250/\text{mm}^3$ , bactDNA quantitation in ascites can sensitively distinguish patients with suspicious infection.

Benzonase has an advantage of removing free DNA fragments without affecting viable bacteria<sup>[24]</sup>. Our data showed that after the removal of free DNA, the copies of DNA detected by ddPCR was generally decreased, while the area under the curve for diagnosing SBP was increased. However, we found that there was a significant decrease in gram-negative bacteria, which we speculated that either the increase of Gram-negative bactDNA from intestinal translocation to abdominal cavity or the increased destruction of Gram-negative bacteria due to the repeated freeze-thaw of samples during storage of ascites samples. Notably, considering the effect of abdominal volume, we found that there was no significant variation of bactDNA between the copies per 1  $\mu\text{l}$  ascites and the total copies of bactDNA combined with ascites volume. Therefore, the dilution of abdominal volume was not a factor affecting the absolute amount of bacteria, which may be due to the high sensitivity of ddPCR detection technology.

Our study showed that the sensitivity and specificity of total bactDNA quantitation was 80.5% and 95.3% compared to the diagnosis of SBP, which was consistent with the results of the bloodstream infection<sup>[12]</sup>. The advantage of ddPCR assay is its culture-independent high sensitivity. It not only obtain the absolute quantification but also accurately distinguish Gram-positive and Gram-negative bacteria. The simple and inexpensive ddPCR process ultimately results in a rapid identification of pathogens within 4 hours. Taken together, the bactDNA quantification in ascites by ddPCR is helpful for dynamically monitoring the changes of nucleic acid in pathogens and the effects of antibacterial treatment.

The SBP diagnosis is confirmed when the ascitic neutrophil count is  $\geq 250 \text{ cells/mm}^3$ . However, in practice, some patients are symptomatic and have a PMN  $< 250 \text{ cells/mm}^3$ . Results from our data showed that those cases had a significant increasing of bactDNA quantification about 2 – 2.5 log copies/ $\mu\text{l}$ , indicating that when PMN counts are not elevated, the bactDNA quantitation improves in detecting bacteria with low loads. And we further observed that PMN counts of  $250/\text{mm}^3$  was probably higher on gram-positive bacteria than gram-negative bacteria. In the future, we will further study the relationship between PMN and the number of bacteria combined the bacteria species, thus further improve the diagnostic efficiency of SBP.

Some potential limitations of this study should be acknowledged. First, One of limitations of using ddPCR with samples is the presence of a small number of false-positive signals in the end point data. Secondly, further study should optimize the extraction method of Gram-negative bacteria and strengthen laboratory asepsis management. Additionally, bacterascites may represent a transient and spontaneously reversible colonization of ascites, or it may represent the first step in the development of SBP<sup>[1]</sup>. The quantification of bactDNA in ascitic fluid by ddPCR may help differentiate the above two scenarios. Further prospective studies should be conducted to bactDNA on the guiding of clinical medication and its prognostic effect in patients with SBP, so as to truly improve the diagnostic value of SBP.

In conclusion, our study shows that the bactDNA quantitation in ascites by ddPCR is a promising approach to improve the diagnostic accuracy of SBP, especially for patients who are symptomatic and have a PMN < 250 cells/mm<sup>3</sup>.

## Abbreviations

SBP: spontaneous bacterial peritonitis. bactDNA: bacterial DNA. PMN: polymorphonuclear count. ddPCR: Droplet digital polymerase chain reaction. CNNA: culture negative neutrocytic ascites; MNB: monomicrobial non-neutrocytic bacterascites; AFI: ascites fluid infection. ROC: receiver operating characteristic. NPV: negative percentage agreement. PPV: positive percentage agreement. NDA: non-dependent assay; BDA: benzonase-dependent assay.

## Declarations

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**Author contributions:** HaoXin Wu, Wei Hou, FeiLi Wei conceived the study, FeiLi Wei, ZhongJie Hu contributed to the study design, HaoXin Wu supervised all aspects of the study, Wei Zhang, Zheng Wang, Zhen Li were responsible for clinical data collection and verification , DeXi Chen, Shan Guo were responsible for results collection and monitoring in the laboratory. HaoXin Wu, Wei Hou wrote the first draft of the manuscript. All authors critically reviewed the manuscript and contributed to writing-editing and approved the final version.

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**Data Transparency Statement:** Statement of data, analytic methods, and study materials will be made available to other researchers.

**Data Availability:** Yes. Data generated or analyzed during this study are available from the corresponding author on reasonable request.

**Animal research (ethics):** Not applicable.

**Plant reproducibility:** Not applicable.

**Clinical trials registration:** Not applicable.

**Conflicts of interest:** Hao-Xin Wu, Wei Hou, Wei Zhang, Zheng Wang, Shan Guo, De-Xi Chen, Zhen Li, Fei-Li Wei and Zhong-Jie Hu authors declare that they have no competing interests.

**Ethical approval:** This study had been approved by Beijing YouAn Hospital, Capital Medical University, Beijing (approval number 176).

**Consent of participate:** All procedures performed were in accordance with the ethical standards of the responsible committee on human experiments and with the Helsinki Declaration of 1975, as revised in 2008.

**Consent to publication:** All authors have read and approved the manuscript.

**Availability of data and materials:** Authors can confirm that all relevant data are included in the article. We agree with the policy in the journal. The data can be shared (or provided) upon reasonable request.

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# Figures

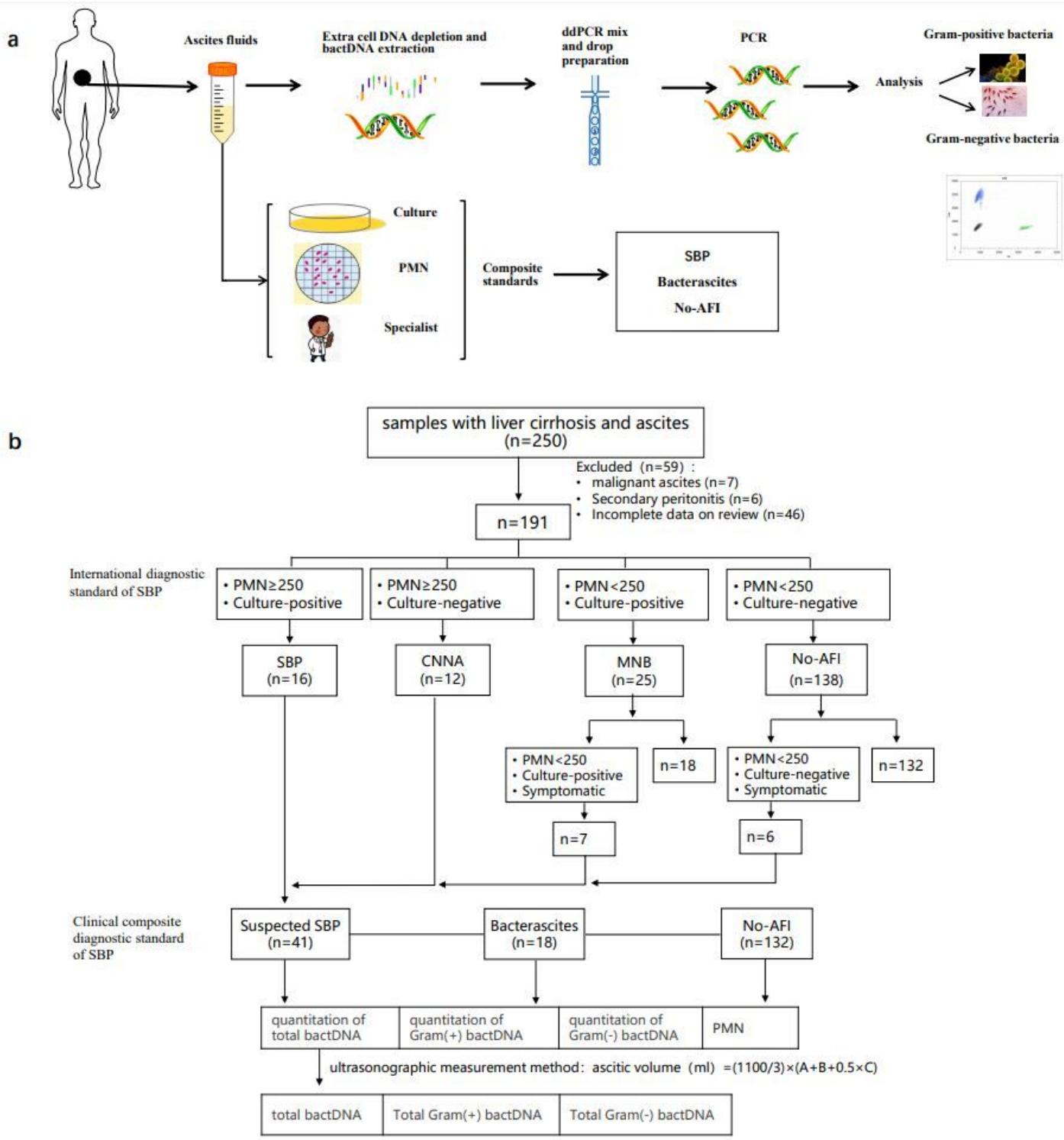
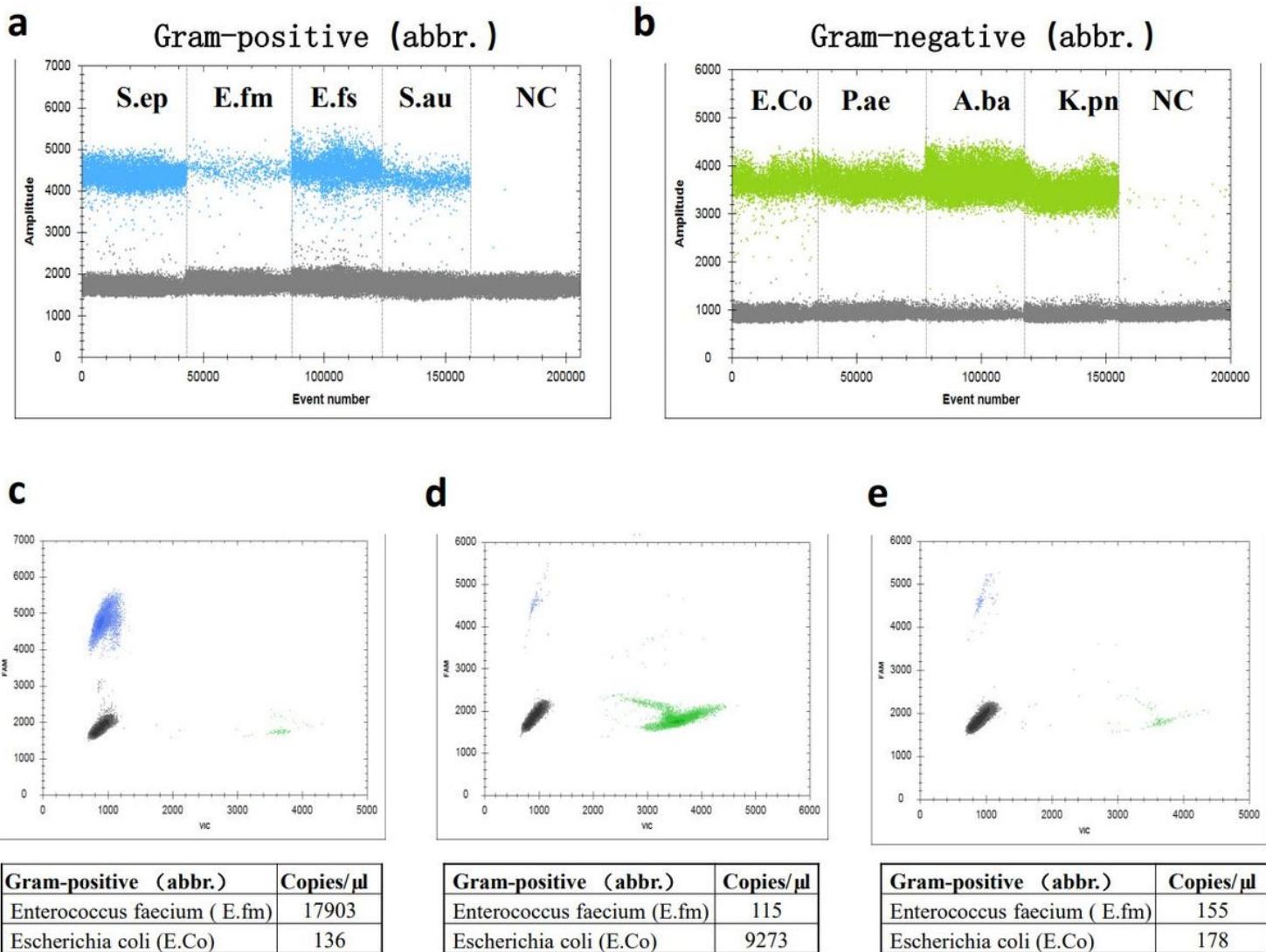


Figure 1

Study workflow and sample distribution.

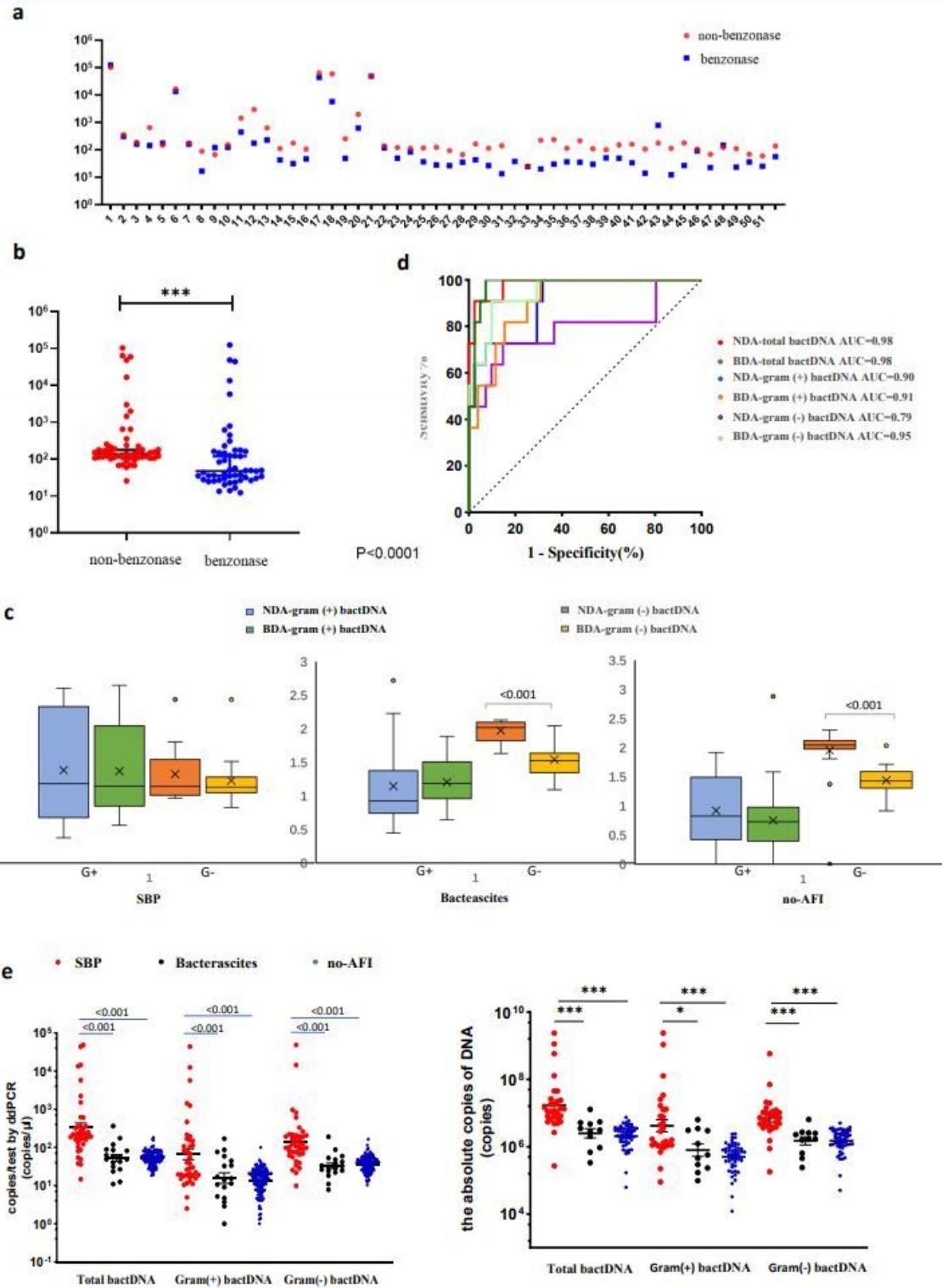
**a.** Schematic of ddPCR ascites fluid analysis workflow. 1.5h-2h were needed for nucleic acid extraction and 2h for the preparation, amplification, and analysis in ddPCR quantitation detection. **b.** Overall flow of patients in the study showing patient recruitment and subsequent selection for bactDNA quantitation analysis.

Abbreviations: ddPCR: droplet digital PCR; PMN: polymorphonuclear; SBP: spontaneous bacterial peritonitis. CNNA: culture negative neutrocytic ascites; MNB: monomicrobial non-neutrocytic bacterascites; AFI: ascites fluid infection.



**Figure 2**

**Detection of Gram-positive bacteria, Gram-negative bacteria using ddPCR.** **a.** Gram-positive bacteria, which included *Staphylococcus epidermidis*, *Enterococcus faecium*, *Enterococcus faecalis*, *Staphylococcus aureus*, and a negative control. **b.** Gram-negative bacteria, which included *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and a negative control. The interference test (different/mixed concentrations) of mixed *E. faecium* and *E. coli*. **c.** 100:1. **d.** 1:100. **e.** 1:1 .

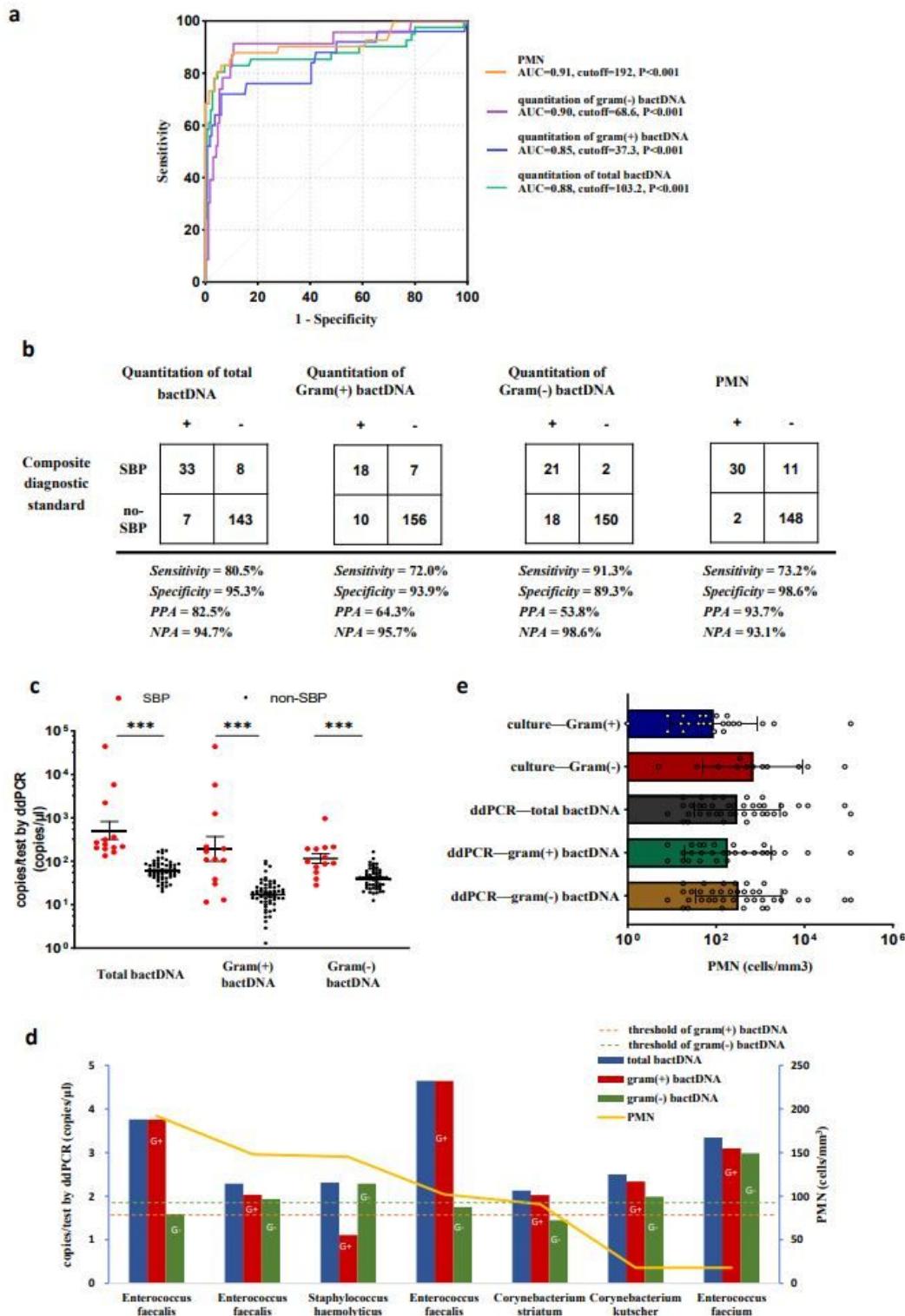


**Figure 3**

**Clinical evaluation of an improved quantitation of ddPCR method.** **a.** 54 patients were selected randomly to this comparation (13 SBP and 41 no-SBP [15 bacterascites, 26 no-AFI samples]). **b.** Comparation with non-dependent assay (NDA) and benzonase-dependent assay (BDA). **c.** The results of gram-positive bactDNA and gram-negative quantitation of NDA and BDA on patients between SBP, bacteascites and no-AFI. **d.** ROC curves stratified by two different methods of NDA and BDA ( $n = 54$  samples in total). Plotted

is the performance of bactDNA based on clinical composite standards. **e**. Samples with 41 SBP, 18 bacterascites, 132 no-AFI. In the left panels, we compared the copies per 1  $\mu$ l ascites volume of DNA quantification. In the right panels, we obtained the total copies of bactDNA combined with ascites volume using virtual ultrasonography “Three-point method”.

Abbreviations: NDA: non-dependent assay; BDA: benzonase-dependent assay. SBP: spontaneous bacterial peritonitis. AFI: ascites fluid infection. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .



## Figure 4

**Accuracy of ddPCR testing in ascites and the bactDNA quantitation in patients with PMN < 250 cells/mm<sup>3</sup>.** **a.** ROC curves of quantification of bactDNA from 191 samples based on clinical composite standards. **b.** 2×2 contingency tables for the validation of the quantification of bactDNA based on a clinical composite standard. **c.** Patients with PMN < 250 /mm<sup>3</sup> (SBP [n = 13] and no-SBP [n = 150]). **d.** The bactDNA quantitation and PMN counts from 7 patients with bacterascites. **e.** PMN counts in 191 samples based on culture and ddPCR.

Abbreviations : ROC: receiver operating characteristic; PPA: Positive predictive agreement; NPA: negative predictive agreement;

## Supplementary Files

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- [Supplementarydata.docx](#)