Does ascitic fluid filtration improve the microbiological diagnosis of spontaneous bacterial peritonitis? An experimental laboratory study

A filtração do líquido ascítico melhora o diagnóstico microbiológico da peritonite bacteriana espontânea? Um estudo experimental laboratorial

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ABSTRACT

Background: Spontaneous Bacterial Peritonitis (SBP) is a serious and frequent complication among cirrhotic patients with ascites and can be diagnosed by cytological analysis of the ascitic fluid. The microbiological culture of ascitic fluid, however, is positive in less than 40% of SBP cases, which often results in inappropriate antimicrobial therapy. Empirical therapy may be suboptimal, increasing patient's risk of aggravation, or overestimated, unnecessarily boosting bacterial resistance. **Objective:** This experimental laboratory study aimed to standardize and verify the technical feasibility of ascitic fluid vacuum filtration, as a way to optimize the etiological diagnosis of SBP, compared to the automated method. Method: The method evaluated and standardized in this study was ascitic fluid vacuum filtration. Its principle is the concentration of bacteria on a filter membrane. **Results:** This study included 36 cirrhotic patients treated at a public university hospital between 11.13.2017 and 06.30.2019. Among them, 47.2% (17/36) presented cytology test results compatible with SBP. For these patients, culture sensitivity using the automated method was 35.3% (6/17), against 11.8% (2/17) with the vacuum filtration method. **Conclusion:** In conclusion, vacuum filtration does not improve the microbiological diagnosis of SBP in this population compared to the automated method.

Keywords: Liver cirrhosis, Peritonitis, Microbiology, Clinical laboratory techniques, Ascitic fluid filtration.

RESUMO

Contexto: A Peritonite Bacteriana Espontânea (PBE) é uma complicação grave e frequente entre pacientes cirróticos com ascite, diagnosticada por meio da análise citológica do líquido ascítico. A cultura microbiológica do líquido ascítico, por sua vez, é positiva em menos de 40% dos casos de PBE, o que resulta frequentemente na instituição de terapia antimicrobiana inapropriada. A terapia empírica pode ser subótima, aumentando o risco de agravamento do paciente, ou superestimada, impulsionando desnecessariamente a resistência bacteriana. **Objetivo:** Estudo experimental laboratorial, propôs padronizar e verificar a viabilidade técnica da filtração a vácuo do líquido ascítico, como forma de otimizar o diagnóstico etiológico na PBE, comparativamente ao sistema automatizado de culturas de sangue. **Método:** O método avaliado e padronizado neste estudo foi a da filtragem a vácuo do líquido ascítico. Esse tem como princípio a concentração da bactéria em uma membrana filtrante. **Resultados:** Nesse estudo, foram incluídos 36 pacientes cirróticos atendidos em um hospital público universitário, entre 13.11.2017 e 30.06.2019. Entre eles, 47,2% (17/36) apresentaram citologia compatível com PBE. Nesses, a sensibilidade da cultura pelo método semi-automatizado foi de 35,3% (6/17) e da cultura pelo método da filtragem a vácuo foi de 11,8% (2/17). **Conclusão:** Em conclusão, a filtragem a vácuo não melhora o diagnóstico microbiológico da PBE em relação ao método automatizado.

Palavras-chave: Cirrose hepática, Peritonite bacteriana espontânea, Diagnóstico microbiológico, Filtragem do líquido ascítico.

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INTRODUCTION

Liver disease contributes significantly to the global burden of disease. Liver cirrhosis accounts for more than 2% of total deaths worldwide [1]. In Southeast and Northeast Europe, an estimated 170,000 deaths per year are attributed to liver disease [2]. In the United States, the mortality attributed to liver disease is about 66,000 deaths per year [3]. In Brazil, liver cirrhosis is the leading cause of hospitalization for liver disease and the eighth attributable cause of death, totaling 308,290 cases [4]. Alcohol is the main etiology of liver cirrhosis [5] and Spontaneous Bacterial Peritonitis (SBP) is the most frequent clinical complication [6].

Ascitic fluid may eventually become infected and evolve into Spontaneous Bacterial Peritonitis (SBP). SBP is a frequent infection in patients with liver cirrhosis, occurring mainly due to bacterial translocation [7]. In terms of clinical diagnosis, SBP is commonly underdiagnosed because of its nonspecific manifestations. In this scenario, cytological analysis of ascitic fluid is an essential resource to confirm SBP. Polymorphonuclear (PMN) counts ≥ 250 cells/mm³ of ascitic fluid establish the diagnosis [8] and are considered a universally accepted cutoff [9].

Microbiological culture is also mandatory for every diagnostic paracentesis and is performed in flasks in automated blood culture systems. Ascitic fluid should be inoculated automated blood culture system at the bedside, at an optimal volume of 10 ml, and prior to the beginning of any antimicrobial therapy. In this way, it is possible to obtain maximum sensitivity for this method [10]. The most commonly isolated bacteria in the ascitic fluid of patients with SBP are members of the *Enterobacteriaceae* family [11]. Microbiological Culture of ascitic fluid is positive in less than 40% of SBP cases [12], which frequently results in the implementation of inappropriate antimicrobial therapies. This low sensitivity causes direct harm to patients, as they receive empirical therapies that contribute to the induction of bacterial resistance.

In this context of low positivity of cultures of SBP patients, the proposition of alternative forms of microbiological diagnosis is justified in order to improve sensitivity, specificity, and support a treatment that is appropriate to the needs of the patient. Given the above, the objective of this study was to verify the technical feasibility of ascitic fluid vacuum filtration and standardize it. Our hypothesis was that the ascitic fluid vacuum filtration method could be more sensitive than the automated method because it can deal with larger volumes of ascitic fluid. Another potential advantage considered was a lower cost, compared to the automated method.

MATERIALS AND METHODS

This is an experimental study conducted at the University Hospital of Ribeirão Preto Medical School, University of São Paulo, Brazil. This article was written in accordance with the STARD checklist guidelines. The sample used was a convenience sample. Cirrhotic adults with clinical suspicion and cytological confirmation of SBP were included in the study. Participants were included upon acceptance and signature of an Informed Consent Form (ICF), either by the patients themselves or their legal guardians. Patients with small unpuncturable ascites were excluded. The study was approved by the Research Ethics Committee of the Centro de Saúde Escola "Joel Domingos Machado" da Faculdade de Medicina de Ribeirão Preto – USP (Joel Domingos Machado Teaching Health Center of the Ribeirão Preto Medical School - USP), Opinion No. 2.198.555.

Paracentesis was performed for every case of recent-onset ascites or according to clinical criteria. A trained physician used an aseptic technique to avoid secondary contamination of the abdominal cavity.

Diagnostic confirmation was possible through absolute polymorphonuclear (PMN) results from total white blood cell counts, both per cubic millimeter and PMN percentage. Counts higher than or equal to 250 PMN per cubic millimeter of ascitic fluid, in the absence of a visible infectious source, establish the diagnosis. For hemorrhagic samples, PMN value was adjusted using the correction factor that subtracts one PMN for every 250 red blood cells per cubic millimeter.

The method evaluated and standardized in this study was ascitic fluid vacuum filtration. Its principle is the concentration of bacteria on a filter membrane. The filter system is composed of a sterile filtration device, filter membrane (Millipore, United States) and vacuum pump (Primar, Brazil). The filter system and membrane are made of polysulfone. Polysulfone is a polymer that is resistant and stable at high temperatures. The filter system consists of a Kitasato and a collecting flask. The vacuum pump

provides a negative pressure of 60kgf/cm², allowing ascitic fluid filtration, which results in a concentration of bacteria on the membrane. A detailed picture of vacuum filtration system and its components (ascitic fluid component, filtration waste and vacuum pump) is shown in FIGURE 1.

In order to ensure the sterility of the filtration system at each use, it was subjected to moist heat sterilization at 121°C for 15 min in an autoclave. The bacterium concentration process was conducted using laminar flow to avoid fluid contamination and performed until system saturation. Saturation can be observed macroscopically by obstructing the passage of ascitic fluid through the system. The membrane was placed at the interface of the Kitasato and the collecting flask using sterile tweezers. Once the technique was performed, the membrane was removed using sterile tweezers and put in contact with the growth medium. Membrane porosity in this analysis was 0.45 µm and the growth medium used was Blood Agar. It must be incubated at 37°C in an Olidef Cz bacteriological incubator (São Paulo, Brazil), and readings should be taken every 24 hours for a maximum of 72 hours. Culture positivity by the filtration technique can be observed by the growth of a single type of bacterial colony within 72 hours.

Figure 1. Vacuum filtration system and its components(ascitic fluid component, filtration waste and vacuum pump)

The reference standard adopted was flask cultivation in an automated blood culture system. It consists of direct inoculation of 10 ml of ascitic fluid at the bedside. After inoculation, it is incubated at 37°C under gentle agitation and continuous monitoring, for up to 7 days in a Bactec® (Becton Dickinson, United States). According to this method, culture positivity is based on bacterial proliferation, resulting in oxygen (O_2) consumption and carbon dioxide (CO_2) release to the medium. The release of CO_2 activates a fluorescence sensor. Once fluorescence is detected, flask positivity is acknowledged.

Ascitic fluid cytology and microbiological results were logged into Research Electronic Data Capture® (REDCap) [13]. General characteristics of the patients were described in frequency tables without further analysis.

To analyze the results of the two methods, we used Sequential Analysis Test [14,15], considering alpha=5%, Beta=20%, and the probability of a positive result of 15% for the reference method and at least 30% for the vacuum filtration method. (Appendix 1)

RESULTS

A total of 36 cirrhotic patients were included in the study, from November 2017 to June 2019. Of these, 17 (47.2%) had SBP-compatible cytology and 4 (23.3%) of them had corrected polymorphonuclear (PMN) values. TABLE 1 shows the clinical and demographic characteristics of the patients included in the study. The patients were mainly men, the median and interquartile range for age were respectively 59,5 and 54 - 68. Alcohol is the most frequent etiology found in cirrhotics patients included in this study. The minority of patients had a previous SBP episode in the last year and Child – Pugh score B was the most common among the studied patients.

The median and interquartile range of total white blood cells per cubic millimeter of ascitic fluid in patients who met the cytological criteria for SBP were 829 and 522-4253, respectively. For absolute values of polymorphonuclear cells per cubic millimeter of

Table 1. Clinical and demographic characteristics of patients included in the study.

Sex	Total (n=36)	%
Male	26	72
Female	10	28
Age range (years)		
30 – 40	1	3
41 – 50	4	11
51 – 60	17	47
61 – 70	8	22
71 – 80	6	17
Aetiology of cirhosis		
Alcohol	11	31
Nonalcoholic steatohepatitis	7	19
Hepatitis C virus + Alcohol	6	17
Others	12	33

Table 1. (final) – Clinical and demographic characteristics of patients included in the study.

Previous Spontaneous Bacterial Peritonitis in the last year	Total (n=36)	%
Yes	8	22
No	28	78
Child - Pugh score		
A	3	8
B	26	72
C	7	20

ascitic fluid, the median and interquartile range were respectively 1186 and 453-3469.

The lowest filtered volume was 20 ml, the highest was 420 ml and the average was 75 ml. Of the 17 (47.2%) patients with SBP-compatible cytology, 3 (17.6%) had the microorganism isolated and identified only by the automated method. While 2 (11.7%) had them isolated and identified by both methods. There was no exclusive isolation and identification of microorganisms by the filtration method. TABLE 2 summarizes the comparative results of the bacterial isolated from ascitic fluid using the automated and filtration methods and having ascitic fluid cytology as the reference standard.

Culture assessment for each method against the reference (cytology) is shown in TABLE 3. The culture obtained using the automated method isolated and identified 6 true positives. The *Bacillus* spp. isolate, although identified in the automated culture, was considered a contaminant. The culture conducted using the filtration method isolated 2 microorganisms considered true positives.

Table 2. Comparative results of the bacterial isolated from the ascitic fluid using the automated method and the filtration method, and having ascitic fluid cytology as the reference standard (n=11)

REDCap® Id	Cytology Result	Microorganism isolated by automated method	Inoculated Volume (ml)	Microorganism isolated by filtration method	Filtered Volume (ml)
1	Negative	*Staphylococcus aureus*	10	*Staphylococcus epidermidis*	40
2	Negative	*Klebsiella pneumoniae*	10	No isolation	100
4	Negative	*Alpha-hemolytic Streptococcus*	10	No isolation	50
11	Negative	*Streptococcus gallolyticus*	10	No isolation	35
12	Negative	No isolation	10	*Moraxella catarrhalis*	60
21	Positive	*Alpha-hemolytic Streptococcus*	10	*Alpha-hemolytic Streptococcus*	250
30	Positive	*Escherichia coli*	10	*Escherichia coli*	20
41	Positive	*Alpha-hemolytic Streptococcus*	10	No isolation	70
49	Positive	*Bacillus* spp.	10	No isolation	20
50	Positive	*Klebsiella oxytoca*	10	No isolation	20
61	Positive	*Enterobacter* spp.	10	No isolation	20

Table 3. Sensitivity and specificity assessment of semi-automated cultures and after ascitic fluid filtration, compared to the reference method (cytology) for SBP diagnosis. (n=36)

Culture/Cytology	Semi-automated culture		Filtration culture	
	positive	negative	positive	negative
Positive cytology	6	11	2	15
Negative cytology	4	15	2	17
Sensitivity	35.30%		11.80%	
Specificity	78.90%		89.50%	

The Sequential Analysis Test identified that the tests were not equivalent, and that the reference method was superior to the filtration method, with 95% of confiability.

DISCUSSION

The ascitic fluid vacuum filtration method for the etiological diagnosis of SBP was standardized and its viability was verified. It was more specific than sensitive compared to the reference method. Despite the low sensitivity, one third of the microorganisms isolated and identified agreed with the ones identified by the automated method. They were in line with the pathogens expected in SBP. This condition was also met by the ones isolated only by the automated method. Regarding cytology, it was suggestive of SBP in almost half of the patients included in the study during the considered period.

Although there is a certain consensus on the inoculation of ascitic fluid at the bedside, the sensitivity of the etiological diagnosis of SBP is usually low [16, 17, 18, 19.]. Efforts have been made to optimize this scenario. In this ways, molecular methods have been proposed as a more sensitive and specific method that does not require inoculate in culture medium. However, these techniques are still expensive nowadays and cannot distinguish colonization from true infection [20,21].

So, in this context, the ascitic fluid vacuum filtration method is an unprecedented and low-cost proposal. In this study, diagnostic sensitivity using the filtration method was 11.8%, against 35.3% for the automated method.

As regards the microorganisms often isolated in ascitic fluid, they have changed over time. This change culminates with an increase in the frequency of isolation of Gram-positive microorganisms, outnumbering gram-negative microorganism isolates [22]. This tendency results from secondary prophylaxis applied to these patients after their first SBP episode. In this work, this observation was compromised due to the low number of cases of SBP patients with suggestive cytology and positive culture. Using the reference method, 2 Gram-positive and 3 Gram-negative microorganisms were isolated. By the filtration method, 1 Gram-positive and 1 Gram-negative microorganism were isolated.

Although the proposed method has a lower sensitivity than the reference method, it is more affordable and allows the reuse of most materials. In order to enable reuse, materials must undergo a sterilization process. Filtration allows a faster identification of the microorganism causing the infection, which can be done directly from the filter membrane, without requiring seeding after its positivity is established. Despite these advantages, the high number of false negatives limits its usefulness in centers that have the resources to use the automated method.

Low sensitivity is possibly due to a low bacterial inoculum with the concomitant presence of immune cells and red blood cells in the fluid. This often results in the prescription of empirical antimicrobial therapies. The use of such therapies leads to the use of broad or narrow-spectrum antibiotics. Broad-spectrum antibiotics have contributed to increased bacterial resistance and accelerated their spread worldwide [23, 24].

CONCLUSION

The present study evaluated the vacuum filtration process of ascitic fluid for the etiological diagnosis of SBP, confirming its technical feasibility. However, the diagnostic sensitivity of filtration was lower than that of the automated method.

APPENDIX 1: Sequential Analysis Test

Considering: Ho ≤ 15%; H1 ≥30%; α = 5%; β = 20%; so P0 = 15%; P = 30% and considering respectively the equation of rejection (R) and acceptance (A):

$$R = \frac{\log\frac{1-\beta}{\alpha}}{\log\frac{P}{P0}} + (n\text{-}s) = \frac{\log\frac{1-P0}{1-P}}{\log\frac{P}{P0}}$$

$$A = \frac{\log\frac{\beta}{1-\alpha}}{\log\frac{P}{P0}} + (n\text{-}s) = \frac{\log\frac{1-P0}{1-P}}{\log\frac{P}{P0}}$$

Replacing these values in the equation we get: R = 2,55 + (n-s) × 0,229 and A = -1,42 + (n-s) × 0,229

And in the tables below are the sample number and the values for success and non-success for each method.

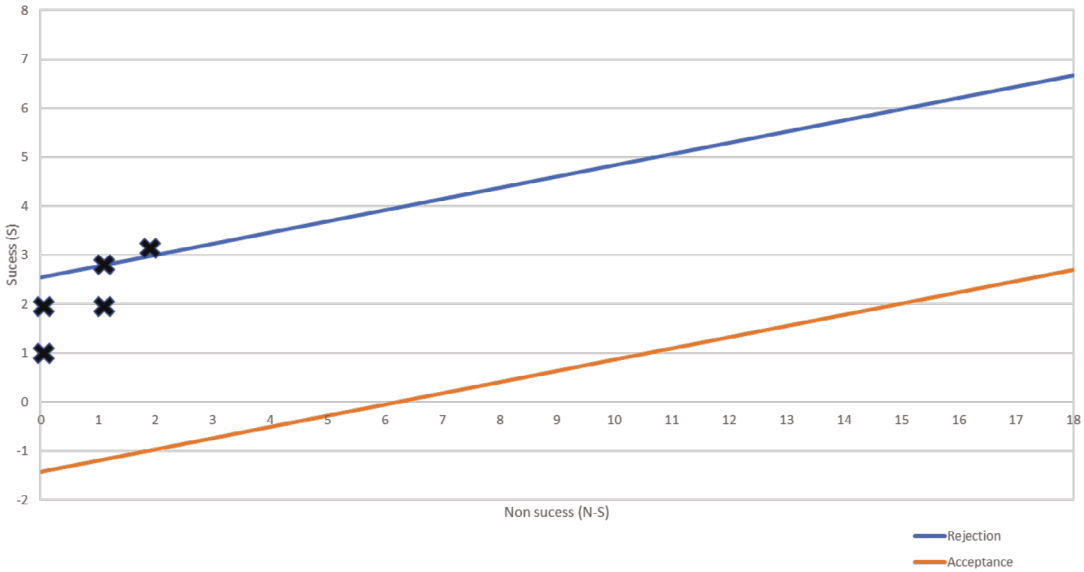
Semi-automated method

Sample number	Success (S)	Non sucess (n-s)
1	1	0
2	2	0
3	2	1
4	3	1
5	3	2

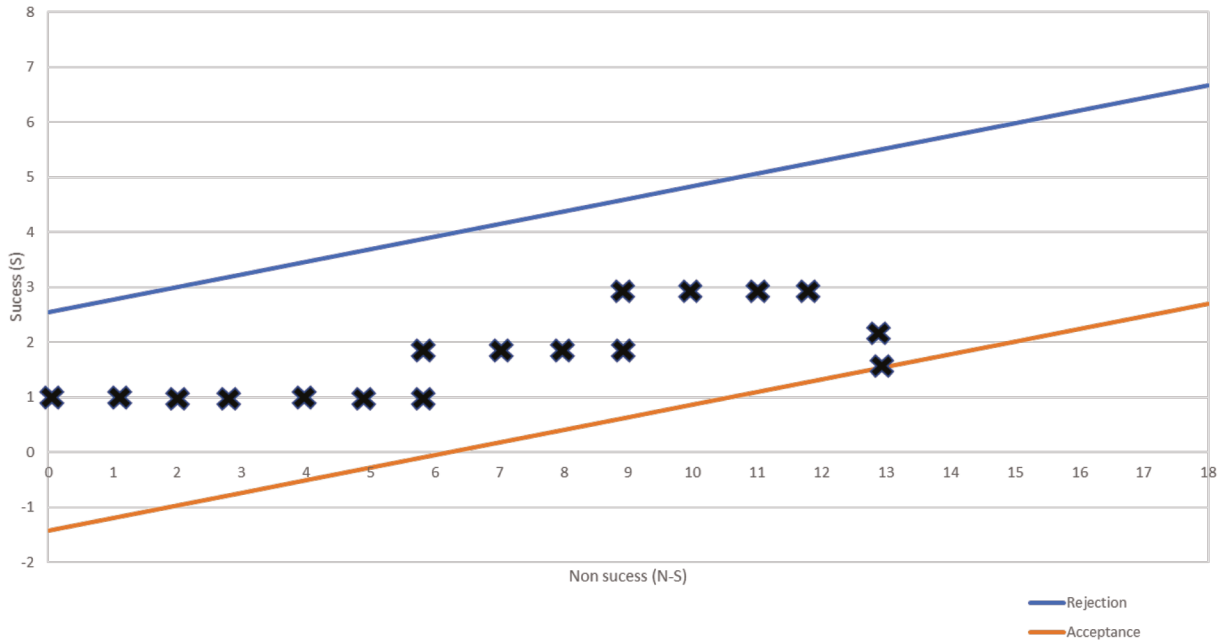
Ascitic fluid vacuum filtration

Sample number	Success (S)	Non sucess (n-s)
1	1	0
2	1	1
3	1	2
4	1	3
5	1	4
6	1	5
7	1	6
8	2	6
9	2	7
10	2	8
11	2	9
12	2	9
13	3	10
14	3	11
15	3	12
16	4	12
17	4	13
18	4	14

Plotting the values for each table we obtain these graphics for **semi-automated method**



And these for **ascitic fluid filtration:**



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Authors' contribution
LC and FBR conceived the study. LC, FBR, FBJ, FFS, RS and RM took part on the study implementation, including patients' inclusion, and data collection. LC wrote the first manuscript draft. All authors critically reviewed the manuscript draft and provided with suggestions. LC and FBR wrote the final version of the manuscript, which has been approved by all authors.

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