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ORIGINAL ARTICLE

Evaluation of Tween 80 incorporated media to increase pathogen isolation from peritoneal fluid of CAPD patients at Hospital Kuala Lumpur

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Abstract

Introduction: Continuous ambulatory peritoneal dialysis (CAPD)-associated peritonitis remains a major complication in patients on CAPD leading to increased morbidity and mortality. Successful therapy of peritonitis is highly dependent on a positive microbiological culture because narrow spectrum antibiotics are essential to efficiently combat infection. Therefore, this study evaluated the performance of Tween 80 containing media at three different concentrations (0.1%, 1.0% and 2.0%) to increase the pathogen yield from peritoneal fluid in comparison with the standard culture media. *Materials and methods:* Peritoneal fluid samples (n=121) obtained from CAPD patients suspected of peritonitis at Hospital Kuala Lumpur were analysed macroscopically and microscopically prior to culture. All samples were cultured on seven different culture media, including sheep blood agar, MacConkey agar, Sabouraud dextrose agar, brain heart infusion agar and Tween 80 incorporated blood agar. All plates were incubated at an optimum temperature up to 48 hours. *Results and conclusion:* Among all the culture media investigated, 0.1% to 2.0% Tween 80 incorporated blood agar yielded the highest positive culture (23/121) in comparison with all other standard media, thus lowering the negative culture rate among CAPD patients. Statistical analysis by Chi Square revealed significant differences ($p < 0.001$) between the three concentrations of Tween 80 tested in this study. Among the three different concentrations of Tween 80 optimised in this study, blood agar containing 0.1% Tween 80 generated the best results, achieved by optimum growth of all Gram-positive organisms, Gram-negative organisms and yeast cells simultaneously. Using a small amount of detergent at low cost significantly increased the pathogen yield during CAPD-associated peritonitis.

Keywords: Tween 80, continuous ambulatory peritoneal dialysis, peritoneal fluid, incorporated media

INTRODUCTION

Peritonitis involves an infection-induced inflammation of the peritoneal layer that covers the inner abdomen and the various organs within the abdomen. This is a frequent complication during episodes of continuous ambulatory peritoneal dialysis (CAPD) contributing to a 16.0% annual death rate among CAPD patients.[1] Peritoneal dialysis-related peritonitis remains the major complication and primary challenge for the long term success of the peritoneal dialysis.[2] Based on the recommendations and guidelines by the International Society for Peritoneal Dialysis (ISPD), peritonitis should be confirmed by a positive microbiological culture.[3] The identification and antibiotic susceptibility profile of the causative pathogen is essential for appropriate drug selection and patient management. ISPD proposed that culture negative peritonitis in CAPD patients should ideally be lower than 20% of all peritonitis

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episodes. However, the negative culture rate in most Malaysian hospitals, as reported by the Malaysian Dialysis and Transplant Registry in 2016 was 24.5%, which is higher than the internationally accepted standards.[4] Lower culture positivity rate among the CAPD patients is usually caused by the presence of antimicrobial agents, a failure to grow intracellular microorganisms and the effect of endotoxins released by bacterial infection.[5] Our previous study has described the outcome in the isolation of bacteria and fungi in CAPD patients with peritonitis using modified laboratory methods including the BACTEC automation identification system and incorporation of 2.0% Tween 80 into blood agar.[6] Tween 80 serves as a non-ionic surfactant that helps to increase the yield of pathogens. The incorporation of Tween 80 into standard blood agar has been described as an option for increasing the yield of clinically significant organisms by disrupting phagocytes and releasing intracellular organisms.[7] Therefore, this study is a continuation effort that aims to evaluate and optimise the performance of Tween 80 containing media at three different concentrations (0.1%, 1.0% and 2.0%) in comparison with the standard culture media to increase the pathogen isolation frequency from peritoneal fluid samples of CAPD patients.

MATERIALS AND METHODS

Sample Collection

One hundred and twenty-one (121) routine peritoneal fluid samples obtained from 121 CAPD patients suspected of peritonitis, between September 2018 to June 2019 at Hospital Kuala Lumpur, were analysed in this study. These samples were collected aseptically in a sterile container and transported immediately at room temperature to the Microbiology Laboratory in the Department of Pathology, Hospital Kuala Lumpur.

Selection Criteria

All 121 peritoneal fluid samples were selected based on the recommendations in the ISPD guidelines, including clinical manifestations such as abdominal pain and fever, high cell counts with WBC >100/mm^3 and polymorphonuclear cell counts of >50.0%, turbid effluents and positive gram stain results. ISPD proposed that the presence of any two of the above conditions including a positive culture can confirm occurrence of peritonitis.[3]

Sample Processing and Analysis

All peritoneal fluid samples received in sterile containers were checked macroscopically for leakage. Sample appearance (turbid, clear, or blood-stained), sample collection date and collection time were recorded. Approximately 15 ml of the peritoneal fluid was centrifuged at 2500 rpm for 20 minutes. The supernatant was discarded, and the sediment was used for cell count, Gram stain and culture.

Media Preparation and Culture

Seven different media types were used in this study for cultivation of peritoneal fluid samples. These samples were cultured directly onto Sheep Blood agar and incubated in aerobic and anaerobic condition at 37°C; MacConkey agar, incubated aerobically at 37°C; Sabouraud dextrose agar and brain heart infusion agar, incubated aerobically at 30°C; Tween 80 (Sigma-Aldrich, USA) incorporated blood agar at 0.1%, 1.0% and 2.0%, respectively, incubated in aerobic and anaerobic conditions at 37°C for 48 hours. All the above culture media were bought commercially (Next Gene, Malaysia) except Tween 80 incorporated media which were prepared in-house. A quality control test with ATCC strains of Gram-positive organisms such as *Staphylococcus aureus* (ATCC 25923), *Streptococcus agalactiae* (ATCC 12403), *Streptococcus pneumoniae* (ATCC 49619), *Enterococcus faecalis* (ATCC 29212); Gram-negative organisms such as *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 70063), *Salmonella* Typhi (ATCC 19430) and yeast, *Candida albicans* (ATCC 10231), were performed on every batch of the media prepared in house.

Isolation and Identification

All plates were incubated for 48 hours but were inspected at 24-hour intervals for growth. Positive isolates were identified using conventional biochemistry such as catalase test, oxidase test, triple sugar iron test, urease test and a microbiological motility test. Some isolates were identified using rapid identification systems including VITEK 2 compact and the API system according to the manufacturer's instructions (bioMérieux, USA).

Statistical Analysis

Chi-Square test was used to compare the positive yield between Tween 80 incorporated media at three concentrations with the standard blood agar.

Ethical approval

This study was approved by both the Malaysian Medical Research and Ethics Committee (KKM. NIHSEC.P18-1174/6) and the Medical Research Ethics Committee of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM/TNCPI/RMC/1.4.18.2 MREC-JKEUPM).

RESULTS

Among the 121 peritoneal fluid samples obtained from CAPD patients during this study, 114 (94.2%) patients had more than two symptoms of peritonitis such as fever, abdominal pain, diarrhoea, and turbid effluent as recommended in the ISPD guidelines. Eighty three (68.6%) samples had a high cell count (WBC >100/ mm^3) and 89 (73.4%) samples were recorded as being turbid or cloudy upon macroscopic examination. However, positive microbiological culture was obtained only in 23 (19.0%) samples. The remaining 98 samples were confirmed as negative by conventional culture methods.

Of the 121 total samples studied, 12 (9.9%) samples showed growth on sheep blood agar, while 11 (9.1%) samples showed growth on brain heart infusion agar, followed by 9 (7.4%) samples showing growth on Sabouraud dextrose agar and 3 (2.5%) samples showing growth on MacConkey agar. However, incorporation of Tween 80 resulted in the highest yields of pathogens in comparison with all other media. Tween 80 incorporation at both concentrations of 0.1% and 1% obtained 19 (15.7%) positive cultures respectively while at 2% Tween 80, 17 (14.0%) positive cultures were successfully obtained (Table 1). Pure growth of one type of bacteria or fungal species was observed in each positive culture obtained and the same specimens showed growth of the same organisms on all positive media.

Table 2 summarises the comparison between Tween 80 incorporated media at three different concentrations (0.1%, 1% and 2%) and standard blood agar without Tween 80 in successful pathogen isolation among the CAPD patients. A Pearson's Chi-square test was conducted and a statistically significant difference (p <0.05) was observed between all three Tween 80 containing media and the standard blood agar. This result revealed that addition of Tween 80 has significantly enhanced the growth and increased the yield of pathogens.

Table 3 shows the type and number of causative agents isolated from the 23 culture positive samples. Among the 23 positive isolates, 16 (69.6%) were Gram-positive organisms, while 5 (21.8%) were Gram-negative organisms and 2 (8.0%) were fungi. Coagulase-negative *Staphylococci* (*CONS*) generated the highest yield (43.5%) in this study, followed by *Staphylococcus aureus* and *Escherichia coli* at 13.2% respectively. Among the Gram-positive organisms, *CONS*, *Staphylococcus aureus* and *Enterococcus faecium* were equally often isolated on all culture media except MacConkey agar. *Streptococcus agalactiae* and *Bacillus* spp. were isolated only on Tween 80 incorporated

TABLE 1: Positivity rate by Tween 80 incorporated media in comparison with other standard culture media (n = 121)

Media	Positive Culture		Negative Culture		Total
	n	(%)	n	(%)	n
Tween 80 incorporated Blood Agar (0.1%)	19	(15.7)	102	(84.3)	121
Tween 80 incorporated Blood Agar (1.0%)	19	(15.7)	102	(84.3)	121
Tween 80 incorporated Blood Agar (2.0%)	17	(14.0)	104	(86.0)	121
Blood Agar	12	(9.9)	109	(90.1)	121
MacConkey Agar	3	(2.5)	118	(97.5)	121
Sabouraud's Dextrose Agar	9	(7.4)	112	(92.6)	121
Brain Heart Infusion Agar	11	(9.1)	110	(90.9)	121

Table 2: Comparison between Tween 80 incorporated media at three different concentrations with standard blood agar in pathogen isolation

		Blood Agar Growth (n=12)	Blood Agar No Growth (n=109)	X^2	df	*p*-value
Tween 0.1%	Growth (n=19)	11 (57.9%)	8 (42.1%)	58.07	1	< 0.05
	No Growth (n=102)	1 (1.0%)	101 (99.0%)			
Tween 1.0%	Growth (n=19)	12 (63.2%)	7 (36.8%)	75.51	1	< 0.05
	No Growth (n=102)	0 (0.0%)	102 (100.0%)			
Tween 2.0%	Growth (n=17)	11 (64.7%)	6 (35.3%)	66.46	1	< 0.05
	No Growth (n=104)	1 (1.0%)	103 (99.0%)			

media. Among the Gram-negative organisms, *Acinetobacter baumanii* and *Escherichia coli* were equally often isolated on almost all of the culture media used. Despite these two Gram-negative organisms, *Moraxella cattarhalis,* which is a less common pathogen in CAPD associated peritonitis, grew only on blood agar, 1.0% Tween 80 incorporated blood agar and brain heart infusion agar. Both medically important *Candida* spp. were isolated only on Tween 80

TABLE 3: Total number of organisms isolated from CAPD associated peritonitis

Organism Isolated	Number (*n*)	Percentage (%)
Gram-positive		
Coagulase -negative Staphylococci	10	43.5
Staphylococcus aureus	3	13.2
Streptococcus agalactiae	1	4.3
Bacillus spp	1	4.3
Enterococcus faecium	1	4.3
Gram-negative		
Acinetobacter baumanii	1	4.3
Escherichia coli	3	13.2
Moraxella catarrhalis	1	4.3
Fungal		
Candida parapsilosis	1	4.3
Candida tropicalis	1	4.3
Total	**23**	**100**

TABLE 4: Distribution of pathogens isolated on different culture media

Organisms isolated	Blood Agar		Blood Agar + Tween 80 (0.1%)		Blood Agar + Tween 80 (1.0%)		Blood Agar + Tween 80 (2.0%)		Mac-Conkey Agar		Sabouraud Dextrose Agar		Brain Heart Infusion Agar	
	n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Gram-positive														
Coagulase-negative *Staphylococci*	**4**	33.3	**9**	47.4	**9**	47.4	**8**	47.1	**0**	0	**1**	11.1	**4**	36.4
Staphylococcus aureus	**3**	25.1	**3**	15.8	**3**	15.8	**3**	17.7	**0**	0	**2**	22.2	**2**	18.2
Streptococcus agalactiae	**0**	0	**1**	5.3	**0**	0	**0**	0	**0**	0	**0**	0	**0**	0
Bacillus spp	**0**	0	**0**	0	**1**	5.3	**0**	0	**0**	0	**0**	0	**0**	0
Enterococcus faecium	**1**	8.3	**1**	5.3	**1**	5.3	**1**	5.9	**0**	0	**1**	11.1	**1**	9.1
Gram-negative														
Acinetobacter baumanii	**1**	8.3	**1**	5.3	**1**	5.3	**1**	5.9	**1**	33.3	**1**	11.1	**0**	0
Escherichia coli	**2**	16.7	**3**	15.8	**3**	15.8	**3**	17.7	**2**	66.7	**2**	22.2	**2**	18.2
Moraxella catarrhalis	**1**	8.3	**0**	0	**1**	5.3	**0**	0	**0**	0	**0**	0	**1**	9.1
Fungal														
Candida parapsilosis	**0**	0	**1**	5.3	**0**	0	**0**	0	**0**	0	**1**	11.1	**1**	9.1
Candida tropicalis	**0**	0	**0**	0	**0**	0	**1**	5.9	**0**	0	**1**	11.1	**0**	0
Total isolate	**12**	100	**19**	100	**19**	100	**17**	100	**3**	100	**9**	100	**11**	100

Table 5: Organisms isolated on Tween 80 incorporated media at different concentrations

Type of Organism	Gram-positive bacteria (*n*=16)		Gram-negative bacteria (*n*=5)		Fungal (*n*=2)	
	n	%	n	%	n	%
Media						
Tween 80 incorporated Blood Agar (0.1%)	14	87.5	4	80.0	1	50.0
Tween 80 incorporated Blood Agar (1.0%)	14	87.5	5	100.0	0	0
Tween 80 incorporated Blood Agar (2.0%)	12	75.0	4	80.0	1	50.0

containing media and fungus selective media, which are Sabouraud dextrose agar and brain heart infusion agar (Table 4).

Table 5 shows the isolation of each type of organism on the three different Tween 80 containing media used in this study. Among the three concentrations tested, the optimum recovery of all types of pathogens which included Gram-positive organisms, Gram-negative organisms and yeasts was obtained on 0.1% of Tween 80. The cost of each agar for one sample was compared in Table 6 and Tween 80 incorporated media were found to be cost-effective with the highest rate of pathogen recovery.

DISCUSSION

Although CAPD is a recognised method for treating end stage renal disease[6], peritonitis remains a major challenge in CAPD which requires prompt and timely medical attention in order to fight the infection. Many new methods have been developed for the identification and isolation of etiological agents from peritoneal fluid samples. However, the specific peritonitis treatment is solely based on the positive culture result as a narrow spectrum antibiotic or antifungal treatment is highly essential.

The recovery of pathogens from CAPD patients with peritonitis lacks sensitivity as the large volume (2 liters) of dialysis fluid in the peritoneal cavity dilutes the pathogen. Only a small amount of peritoneal fluid is sent to the laboratory for culture and microscopy. The efficacy of the standard culture media being used in current isolation methods to enhance pathogen yield is still low. As an alternative method, many studies have demonstrated the effect of Tween 80 on bacterial growth. One study has reported that the addition of 0.1% Tween 80 to culture media increased the growth rate of planktonic *Staphylococcus aureus* batch culture.[8] A recent study by Iyer *et al*. (2014)[7] documented that the addition of 2% Tween 80 resulted in additional culture positivity compared to the direct conventional culture method of peritoneal fluids. In this study, for the first time, three different concentrations were optimized to obtain a better yield of pathogens from CAPD patients.

Table 6: Cost Comparison between different culture medium

Media (*n*=1)	Cost (RM)
Sheep Blood Agar	1.25
MacConkey Agar	1.70
Sabouraud Dextrose Agar	1.45
Brain Heart Infusion Agar	1.90
Tween 80 incorporated blood agar	1.40

We found that Tween 80 containing media at all three concentrations achieved higher yields ($p<0.001$) of pathogens in comparison with other standard media. Even though the current isolation method for peritonitis agents at Hospital Kuala Lumpur prescribes only standard blood agar and MacConkey agar, other fungal selective media such as brain heart infusion agar and Sabouraud dextrose agar were added into our study to support the growth of yeast. This study revealed that the occurrence of Gram-positive bacterial peritonitis (69.6%) was the highest followed by Gram-negative bacterial peritonitis (21.8%) and fungal peritonitis (8.6%) in CAPD patients.

Our findings recorded CONS as the most frequent isolate (10/23) which is in accordance with the recent 24th report of Malaysian Dialysis and Transplant Registry (2016)[4] that reported coagulase negative *Staphylococcus* (CONS) as a leading causative agent in peritonitis. The increase rate of Gram-positive bacterial peritonitis in CAPD patients could occur due to invasive procedures during catheter insertion in the peritoneal cavity or contamination by skin.[9]

In this study, we encountered a rare causative agent in peritonitis, *Moraxella catarrhalis,* in a 54-year-old CAPD patient presenting with fever, diarrhoea and turbid effluent. A similar case has been reported in 2012 in a 56-year-old CAPD patient with end stage renal disease.[10] Fungal peritonitis, although a rare scenario in CAPD-associated peritonitis but with 20-30% mortality rate, has an incidence of up to 4% in CAPD peritonitis globally.[11] One study by Ahamed and Vermette (2009)[12] reported that the increased cell permeability caused by surfactant could enhance the enzyme activities in yeast and promote the growth. In our study, only Tween 80 containing media at 0.1% and 2.0% along with fungal selective medium were able to promote growth of both *Candida parapsilosis* and *Candida tropicalis* while the standard blood agar without Tween 80 failed to produce any within 48 hours of the incubation period.

The effectiveness of non-ionic surfactant agents such as Tween 80 in increasing the pathogen recovery from body fluids has been described in many studies. The commercial Tween 80 solution contained some oleic acid which has been proven to inhibit bacterial adhesion and biofilm formation of *Staphylococcus aureus* by hydrolysing fatty acid ester bonds.[13] Tween 80 lyses the leukocytes and macrophages to allow the intracellular organisms to grow on the culture media. Intracellular organisms will be amenable to growth only when they are released from PMN cells by the surfactant. Tween 80 as a surface-active substance, is also believed to promote the dispersion of the viable cells thus increasing the growth rate.[14]

Based on the clinical history of the CAPD patients, two patients out of 23 culture positive peritoneal fluids were treated with antibiotics. Both patients were given intravenous (IV) cloxacillin prior to sample collection. However, coagulase-negative *Staphylococcus* (CONS) and *Staphylococcus aureus* were successfully cultivated using 0.1% Tween 80 containing media as it is believed to block the effect of cloxacillin in these two patient's peritoneal fluid samples. This finding is in accordance with one previous study in 2014 that explained the antibiotic potency of platensimycin and triclosan as fatty acid biosynthesis inhibitors were completely abolished at concentration as low as 0.02% up to 0.1% of the Tween 80 concentrations.[15]

In this current study, defibrinated bovine blood was used and we found that its red blood cells composition was similar to horse blood. However, bovine blood is much easier to obtain and cheaper compared to horse blood that was utilised in the previous study.[6] Based on the cost analysis calculated for the powder and raw materials illustrated in Table 6, a routine microbiology laboratory needs a combination of three to four standard culture media which will cost around RM6.30 per sample for a successful isolation of both bacteria and fungi. However, usage of Tween 80 containing media remains the best isolation method of peritonitis agents at a cheaper cost of RM1.40 per sample. Nevertheless, some reports stated the limitation of Tween 80 in having growth inhibitory effect on certain bacteria such as *Listeria monocytogenes* and *Pseudomonas fluorescens*.[8] Therefore, usage of Tween 80 incorporated media in the diagnosis of peritonitis may not be the alternative that replaces the use of other routinely used media. It surely will complement the current media portfolio of laboratories that cannot afford the use of commercial and relatively expensive blood culture systems.

CONCLUSION

In conclusion, this study shows that the incorporation of Tween 80 at concentrations between 0.1% to 2.0% provides a cost-effective method generating higher yields of pathogens in peritoneal fluid obtained from CAPD patients in comparison with other standard culture media.

However, among the three concentrations tested, 0.1% Tween 80 seems to be the best concentration to support the optimum growth of all Gram-negative and Gram-positive organisms and yeast. This approach may be applied in laboratory diagnostics, particularly in resource-limited settings to enhance the yield of pathogens in CAPD associated peritonitis.

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Authors' contribution: SGP, SAN, SNM and FA designed the study. SDWMS contributed to the data collection. SGP performed testing, analyzed data and prepared the manuscript. SAN, SNM, FA and NMJ supervised the study. AVB and SAN reviewed and edited the manuscript. All the authors read and approved the final version of the manuscript.

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