CHARACTERIZATION OF THE PERITONEAL FLUID BACTERICIDAL TITRE (PBT) FOR USE IN THE TREATMENT OF CONTINUOUS AMBULATORY PERITONEAL DIALYSIS RELATED PERITONITIS.

BY

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Characterization of the Peritoneal Fluid Bactericidal Titre (PBT) for Use in the Treatment of Continuous Ambulatory Peritoneal Dialysis Related Peritonitis

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

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ABSTRACT

Purpose: MICs, which fail to assess factors such as intraperitoneal (IP) antibiotic concentrations and dianeal fluid effects on antibacterial activity are poor indicators of clinical outcome in the treatment of peritoneal dialysis- related peritonitis. The purpose of the study was to develop a new microbiologic test in spent dialysate called the peritoneal fluid bactericidal titre (PBT). **Method:** Simulated 6h and 24h IP concentrations of cefazolin plus tobramycin achieved with empiric IP treatment once daily and IP cefazolin once daily were studied. Six dianeal fluids and 36 peritoneal isolates (i.e., six of *S.epidermidis, S.aureus, E.cloacae, E.coli, K.pneumoniae, P.aeruginosa*) were tested. Complete biochemical analyses were performed on all dianeal fluids. Dianeal fluids were processed, serially diluted with broth and inoculated to yield 1 x 10^6CFU/ml. After a 24 h incubation (35°C), inhibitory and bactericidal titres were determined. PBTs related to species and dianeal fluids were examined. A pilot study was also preformed in 14 patients using their spent dianeal fluid, infecting organism and clinical outcome data. Paired t-tests and the Mann-Whitney tests were preformed where appropriate to determine statistical significance. **Results and Conclusions:** PBTs were reproducible with characteristic ranges for different organisms. PBTs detected differences in antibacterial activity between dianeal fluids. PBTs at 6h antibiotic concentrations for cefazolin plus tobramycin were greater than PBTs at 24h concentrations. PBTs for cefazolin plus tobramycin were greater than PBTs for cefazolin alone. Cefazolin plus tobramycin PBTs were highest for *S. aureus* (median of 1/128 at 6h and 1/64 at 24h) and lowest for *P.aeruginosa.* (median of 1/48 at 6h and 0 at 24h) The pilot study suggested a significant association between PBTs and clinical outcome. ($p= 0.036$)

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INTRODUCTION

The development of continuous ambulatory peritoneal dialysis has improved the lives of many people living with kidney failure. This form of dialysis is advantageous in many aspects and can be done at home providing greater lifestyle freedom. Unfortunately peritonitis is a significant cause of morbidity and mortality in patients on continuous ambulatory peritoneal dialysis (CAPD). (Troidle, Gorban-Brennan et al. 1998) Peritonitis is an inflammation of the membrane, called the peritoneum, which lines the inside of the abdomen and all of the internal organs. It occurs at a rate of 0.5 episodes/patient/year and can lead to significant complications requiring catheter removal and, in some cases, transfer to hemodialysis. (Zelenitsky, Barns et al. 2000) CAPD-related peritonitis is most commonly treated with the intraperitoneal administration of antibiotics.

Dianeal fluid has been shown to alter antibiotic activity against organisms that cause peritonitis. Spent dialysate, which has been drained from the peritoneum after a dwell period varies in biochemical composition and its ability to support bacterial growth. Standard microbiology tests of antibiotic sensitivity, such as minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) are assessed in standard media. This project focused on the development of a new test called the peritoneal fluid bactericidal titre (PBT) which measures antibiotic activity in spent dialysate collected from individuals with peritonitis. It assesses antibiotic activity within the peritoneal environment. Another advantage of the PBT is that it measures total antibacterial effect of combination antibiotics. This is especially important for CAPD-related peritonitis, which is often treated with two antibiotics. PBTs can be used to assess

and compare different antibiotic regimens. PBT can also provide information in individual cases to tailor the treatment of peritonitis and optimize clinical outcome.

Patients presenting with signs and symptoms of peritonitis are started on empiric treatment regimens. Empiric treatment regimens cover the most commonly isolated pathogens associated with CAPD peritonitis. Due to the relatively small CAPD population, clinical studies with sufficient sample sizes are difficult to conduct. As a result, treatment recommendations are often made without solid clinical evidence. The most current International Society for Peritoneal Dialysis (ISPD) 2000 guidelines for empiric treatment switched from intraperitoneal cefazolin and tobramycin once daily to intraperitoneal cefazolin and ceftazidime once daily. Although there is no clinical data on the efficacy of ceftazidime in empiric treatment, the change to ceftazidime was recommended in order to reduce routine exposure to aminoglycosides so as to preserve residual renal function in CAPD patients.

The use of a peritonitis specific microbiology test such as the PBT would contribute significantly to the evaluation and comparison of antibiotic therapies for this indication.

REVIEW OF LITERATURE

A. CONTINUOUS AMBULATORY PERITONEAL DIALYSIS

Continuous ambulatory peritoneal dialysis (CAPD) is a form of dialysis used in patients with end stage kidney disease. A thin membrane that surrounds the intestines and internal organs called the peritoneum lines the peritoneal cavity. CAPD functions though continuous filtration within the peritoneal cavity to remove wastes from the blood and excess water. During CAPD, a bag of dianeal fluid, about 2L, is drained into the peritoneal cavity through a surgically implanted catheter. The fluid is left in the body for 4 to 6 h, while it absorbs waste products from the blood, through the peritoneum, which acts as a filter. The dwell period is defined as the amount of time the dianeal fluid is left in the peritoneum. Following a dwell period, the dianeal fluid is then drained out and replaced with fresh dianeal fluid. Dianeal fluid drained from the peritoneum following a dwell is termed spent dianeal fluid. The exchange process is spaced out throughout the day and repeated four to six times a day.

Dianeal fluid contains varying amounts of dextrose ranging from 0.5% to 4.25%. Dextrose present in dianeal fluid (Dianeal ®) acts as an osmotic agent to remove excess fluid. During a dwell period dextrose may be absorbed systemically depending on the absorption characteristics of the peritoneal membrane and the patient's blood glucose level. Dianeal fluid also contains added amounts of calcium chloride, magnesium chloride, sodium chloride and sodium lactate. Another type of dianeal fluid called

Extraneal ® contains 7.5% icodextran and is used in patients who require greater fluid removal. Icodextran based solutions have been shown to be more biocompatible than glucose based solutions, thereby extending treatment life for CAPD patients. Nutrineal® is another type of dianeal fluid which consist of an amino acid based solution used to provide extra nutrition to CAPD patents suffering from malnutrition.

B. CAPD-RELATED PERITONITIS

Peritonitis is a major complication for patients undergoing CAPD, and occurs at a rate of 0.5 episodes/patient/year. (Keane, Bailie et al. 2000; Zelenitsky, Barns et al. 2000) The incidence of CAPD peritonitis has declined dramatically in the past ten years primarily due to catheter connection devices, which reduce the risk of infections due to skin organisms. (Keane, Bailie et al. 2000) Complications of peritonitis include hospitalization, catheter removal and subsequent transfer to hemodialysis in 20 to 30% of cases. (Bailie and Eisele 1995)

1.DIAGNOSIS

Patients with peritonitis can present with cloudy dialysate fluid, abdominal pain and fever. A review of peritonitis symptoms showed abdominal pain was present on admission in 79% of cases and temperature >37.5^{0}C in 53% of cases. (Vas and Oreopoulos 2001) Patients presenting with these symptoms will have spent dianeal fluid (minimum dwell period of 4h) sent for cell count, Gram stain and culture. Gram stain

results are positive in 9-40% of cases and are predictive of positive culture results 85% of the time. (Keane, Bailie et al. 2000)

The ISPD 2000 guidelines recommend prompt initiation of antibiotic treatment for a patient with cloudy dianeal fluid, abdominal pain and/or fever and when dialysate white blood cell count (WBC) is >100/ mm^3 with ≥50% polymorphonuclear neutrophils (PMN). If cloudy dianeal fluid is the only symptom, it is reasonable to obtain cell count and Gram stain results (usually two-three hours) before starting antibiotic therapy. If there is no elevation in WBC count, no predominance of PMN and no organisms seen on Gram stain immediate antibiotic therapy is usually unnecessary.

2. ETIOLOGY

1. Sources of peritoneal infections

The major source of peritonitis is skin contamination with endogenous flora, which accounts for 50 to 60% of all peritonitis infections. (Vas and Oreopoulos 2001) The causative organisms usually consist of *Staphylococcus epidermidis, Acinetobacter, Staphylococcus aureus, Pseudomonas* and yeast. The second most common source of peritoneal contamination is from intestinal flora, which account for 25-30% of peritonitis cases. Infections from this source usually consist of enteric Gram-negative organisms and anaerobes. (Vas and Oreopoulos 2001)

2. Gram-positive infections

Gram-positive organisms are the most frequently isolated pathogens in 60% to 80% of all peritonitis cases. (Bailie and Eisele 1995; Zelenitsky, Barns et al. 2000) *S. aureus* and *S. epidermidis* are the most common causes in approximately 30% and 20% of infections, respectively. (Zelenitsky, Barns et al. 2000) (Troidle, Gorban-Brennan et al. 1998) (Vas and Oreopoulos 2001)

3. Gram-negative infections

Gram-negative bacteria are isolated in 20% to 35% of all peritonitis cases. (Vas 1983; Zelenitsky, Barns et al. 2000) (Bailie and Eisele 1995) *Pseudomonas aeruginosa*, *Escherichia* coli and *Klebsiella* species are the most common causes of Gram-negative peritonitis and are isolated in approximately 7.1%, 6.8% and 5.2% of infections respectively. (Zelenitsky, Barns et al. 2000) A study by Bernardini et al. isolated *P. aeruginosa* in 5% of all peritonitis cases.

3. TREATMENT

1. Empiric Therapy

Patients with suspected peritonitis are started on empiric antibiotic therapy and depending on the severity of infection may be admitted to hospital. Most treatment

involves the intraperitoneal administration of antibiotics with or without concurrent systemic antibiotics. Antibiotics are added to about 2L of dianeal fluid, which is drained into the peritoneum for a specific dwell period. Once patient-specific microbiology results have been obtained, therapy is then tailored to the pathogen and its antibiotic sensitivity profile.

Empiric therapy from the ISPD 1996 guidelines switched from intraperitoneal administration of vancomycin once weekly plus aminoglycoside once daily to a first generation cephalosporin plus aminoglycoside once daily. (Keane, Alexander et al. 1996) An increase in vancomycin resistant microorganisms, mainly seen in enterococci, prompted a reduction in the use of vancomycin as empiric therapy. Current empiric therapy at the St. Boniface Hospital Dialysis clinic (Winnipeg, Canada) consists of intraperitoneal cefazolin 1.5g / 2L (1g if < 50kg) plus tobramycin 60mg / 2L (40mg if <50kg) once daily with a minimum 6 h dwell period. A single IP dose of 1.5 g of cefazolin produces average dialysate concentrations of 116 ug/ml at 6h (i.e. end of first dwell period) and a concentration of 32 ug/ml after 24h (i.e. end of 4th dwell period). (Low, Gopalakrishna et al. 2000) Intraperitoneal administration of once daily tobramycin 2 mg/kg with 6h dwell periods produces average dialysate concentrations of approximately 20 ug/ml following a 6h dwell and 3.2 ug/ml at 24h. (Walshe, Morse et al. 1986)

Empiric therapy from the most recent ISPD 2000 guidelines consists of intraperitoneal administration of a first-generation cephalosporin (1g once daily) plus

ceftazidime (1g once daily). The switch to ceftazidime as empiric therapy was to avoid aminoglycoside nephrotoxicity and preserve residual real function, an independent predictor of patient survival. (Keane, Bailie et al. 2000) Ceftazidime was selected due to its activity against gram-positive and gram-negative organisms as well as activity against *Pseudomonas* species. Due to concerns of ceftazidime resistance and lack of supporting clinical data, the use as ceftazidime as empiric therapy at the St. Boniface Hospital has not been adopted. The ISPD 2000 guidelines recommend the use of cefazolin in combination with aminoglycoside, clindamycin or vancomycin in patients with residual urine volume <100 ml/day.

2. Gram-Positive Peritonitis (ISPD recommendations)

If the organism is identified as *Enterococcus,* empiric therapy is discontinued and ampicillin 125mg/L is added to each bag of dianeal fluid for a total of 14 days. The routine use of vancomycin for the treatment of enterococci is discouraged and therapy with vancomycin is recommended only for enterococci isolates that demonstrate ampicillin-resistance.

If the organism is identified as *S.aureus*, the first generation cephalosporin is continued and ceftazidime or aminoglycoside is discontinued. If there is no improvement in symptoms in 24- 48h, oral rifampin may be added for a total of 21 days. If the organism is identified as methicillin-resistant *S. aureus* a switch to vancomycin 2g per 2L of dianeal fluid every 7 days or clindamycin with oral rifampin for 21 days is

recommended. Intraperitoneal administration once weekly of vancomycin 30 mg/kg with 6h dwell periods produces average dialysate concentrations of approximately 610 ug/ml following a 6h dwell and 5 ug/ml at 24h. (Morse, Farolino et al. 1987)

For all other gram-positive organisms including coagulase-negative staphylococcus the first generation cephalosporin is continued for a recommended total 14 days, with the discontinuation of ceftazidime or aminoglycoside. If the organism is identified as methicillin-resistance *S.epidermidis* and the patient is not responding clinically to treatment after 48 h a switch to either vancomycin or clindamycin may be warranted.

3. Gram-Negative Peritonitis (ISPD recommendations)

If *E.coli* or *Klebsiella* is identified and demonstrates sensitivity to ceftazidime, therapy may be continued with ceftazidime for 14 days and the first generation cephalosporin may be discontinued. According to the most current guidelines, if residual urine output is <100ml/day an aminoglycoside may be added. If the patient is on empiric therapy consisting of a first generation cephalosporin plus aminoglycoside both antibiotics are continued. If the isolate is identified as *Pseudomonas*, ceftazidime is continued and a second agent such as piperacillin IV, oral ciprofloxacin (if >100ml urine/ day), intraperitoneal aminoglycoside (if <100ml urine/day) or sulfamethoxazole/trimethoprim be added for a total of 21 days. Current *Pseudomonas* therapy at the St. Boniface Hospital Dialysis clinic, (Winnipeg, Canada) consist of

ceftazidime 125mg/L administered into each bag plus tobramycin 60mg (40mg if <50kg) once daily with a minimum 6-h dwell period. Repeated administration of ceftazidime 125mg/L into each dialysate bag with each exchange will result in an average dialysate concentration of 30ug/ml following the initial 6h dwell and 43ug/ml at 24h (i.e. end of 4th dwell period). (Ryckelynck, Vergnaud et al. 1986)

4. TREATMENT OUTCOMES

Treatment of peritonitis varies from center to center and there are limited data comparing the efficacy of treatment regimens. Treatment failure is defined as recurrent infection within 14 days, catheter removal, transfer to hemodialysis or death.

1. Gram-positive peritonitis

The once daily cefazolin and aminoglycoside regimen has treatment failure rates of 30% for gram-positive peritonitis. Peritonitis caused by coagulase-negative staphylococci, particularly *S. epidermidis* are generally less severe and respond well to appropriate antibiotic treatment with symptoms resolving usually within two to three days. (Vas and Oreopoulos 2001) Infections cause by *S. aureus* are much more severe, causing hypotension, severe abdominal pain and in some cases shock. *S.aureus* peritonitis also responds well to treatment although symptoms usually last longer especially when residual abscesses are present. Failure rates for *S. aureus* and coagulase

negative staphylococcus infections are 29% and 20% respectively. (Goldberg, Clemenger et al. 2001)

2. Gram-negative infections

Gram-negative peritonitis is associated with greater severity and consequently greater risk of mortality. (Fried, Bernardini et al. 1996) For Gram-negative infections, treatment failure occurs in approximately 30% of cases. (Piraino, Bernardini et al. 1987; Lye 1999; Goldberg, Clemenger et al. 2001) Peritonitis caused by *Pseudomonas* species can be difficult to treat due to the formation of multiple abscesses or formation of biofilms on the catheter, which limit antibiotic penetration. In most cases double antibiotic coverage is required as well as catheter removal. (Vas and Oreopoulos 2001) Treatment failure rates for *Pseudomonas* species can be much higher and range from 20 to 50%. (Piraino, Bernardini et al. 1987; Szeto 2001)

C. MIC/ MBC

1. DESCRIPTION

MICs are determined by broth or agar dilution methods and are used to measure the in vitro antibiotic activity. MIC evaluation is based on guidelines provided by the National Committee on Clinical Laboratory Standard (NCCLS). Tubes of broth, which contain serial dilutions of antibiotic concentrations, are used to measure antibiotic activity

against a particular bacterial isolate. The endpoint MIC is determined by visual inspection of each tube and is read as the lowest concentration, which demonstrates an inhibition of growth. (i.e.: first visibly clear tube) The MIC value is used as an indication of antibiotic concentration required at the site of infection. The MIC of an antibiotic is further classified as sensitive, intermediate or resistant.

NCCLS breakpoints are based on average antibiotic serum concentrations achieved with standard doses. An organism classified as susceptible can be appropriately treated with a standard doses of corresponding antibiotic. Therefore if the MIC for an isolate is below the breakpoint value, the organism is considered sensitive and the antibiotic is considered appropriate for treatment. Intermediate strains consist of MICs that depict levels that may be achieved but with higher dose ranges. A resistant strain is predicted to be insufficiently inhibited by the concentrations achieved with normal antibiotic doses. (NCCLS 2000)

MBCs measure the in vitro bactericidal activity of an antibiotic. Bactericidal activity by definition is a 3log kill or a 99.9% reduction in bacterial inoculum. The MBC endpoint is determined by the amount of bacterial kill in each serially diluted tube and is read as the lowest concentration capable of a 3log kill or a 99.9% reduction in bacterial inoculum.

D. ISSUES IN THE TREATMENT OF CAPD PERITONITIS

1. DIANEAL FLUID

Composition of spent dianeal fluid varies in pH, glucose, protein and immune factors. Following intraperitoneal administration, the pH of dianeal fluid will neutralize and urea, creatinine, amino acids and protein will dialyze out into the dianeal fluid. Prior to instillation, fresh dianeal fluid is acidic and has an approximate starting pH of 5. Within 1 h of instillation, dianeal fluid will gradually neutralize to an approximate pH of 7. The pH of spent dianeal fluid when exposed to air and incubated in vitro at 37°C will also increase by a mean of 1.23U and precipitation of calcium, magnesium, phosphate and proteins can occur. (Wilcox, Smith et al. 1990) Inflammation of the peritoneal membrane during peritonitis will increase transport across the membrane and can result in greater protein loss. (Levey and Harrington 1982)

Staphylococci cannot survive in fresh dianeal fluid. (Verbrugh, Keane et al. 1984) (Sheth, Bartell et al. 1986) MacDonald et al. observed no growth of *S. epidermidis* in fresh dianeal fluid regardless of changes in glucose concentrations and neutralization of dianeal fluid. Spent dianeal fluid provides growth media for most bacterial organisms following a dwell period of 4 to 6 hours within the peritoneal cavity. Components present in spent dialysate such as serum proteins and amino acids promote the growth of *S.epidermidis* and *S.aureus*. (Verbrugh, Keane et al. 1984; McDonald, Watts et al. 1986) *S.epidermidis* and *S. aureus* are capable of growth in spent dianeal fluid and have

exhibited six to 13 fold increases in cell counts within 24h, respectively. (Verbrugh, Keane et al. 1984) Studies indicate that factors such as acidic pH and phenotypic changes to cell surfaces can also significantly alter growth characteristics. (McDonald, Watts et al. 1986; Wilcox, Smith et al. 1990) (Wilcox, Edwards et al. 1985) Changes in pH with variations in dwell times do not significantly affect *S. epidermidis* growth. (McDonald, Watts et al. 1986) Sheath *et al.* observed growth for *S. epidermidis* and *S.aureus* in spent dianeal fluid up to 48 h, however there was poor organism survival in spent dianeal fluid beyond 96h. Calcium levels in spent dialysate may also affect coagulase-negative staphylococci growth. Coagulase-negative staphylococci grow less well in spent dialysate when calcium concentrations are at physiologic concentrations, although some stains have demonstrated better growth at lower calcium dialysate levels. (Morton, Evans et al. 1994) *E.coli* is capable of growth in both fresh and spent dianeal fluid. (Verbrugh, Keane et al. 1984) *E.coli* and *P.aeruginosa* have demonstrated greater than 1000-fold increases in cell counts in spent dianeal fluid. (Sheth, Bartell et al. 1986)

The presence of dianeal fluid within the peritoneal cavity also has inhibitory effects on cellular defense mechanisms. (McDonald, Watts et al. 1986; Thomas, Schenk et al. 1997) In peritonitis, inflammation of the peritoneum causes an increase in the number of PMN cells within the peritoneal cavity. These cells act by phagocytosis to remove bacteria from the peritoneal cavity. When administering a large amount of dianeal fluid into the peritoneal cavity, the low pH and high osmolarity of the dialysate has been shown to decrease the efficiency of phagocytosis. (Duwe, Vas et al. 1981) Studies have demonstrated that glucose polymer dianeal fluids, such as Icodextran® are

less osmotic and may be less suppressive to phagocytic activity within the peritoneal cavity. (Thomas, Schenk et al. 1997)

2. ANTIBIOTICS

1. Pharmacokinetics of intraperitoneal administration

In the treatment of CAPD peritonitis, antibiotics are added to one exchange of dianeal fluid once daily for a dwell period of four to six hours. There are limited studies examining intraperitoneal antibiotic administration and the pharmacodynamic activity that occurs within the peritoneal cavity. Antibiotic transport and clearance is dependent on the peritoneal membrane transport status of individual patients. The movement of antibiotic from the peritoneal cavity into the systemic circulation is dependent on the duration of the dwell period and on differences in concentration gradients between the dialysate and blood. Once daily intraperitoneal antibiotic administrations is preferred because of therapy simplification and increased compliance. Within the first dwell period there is a linear decline in dialysate antibiotic concentration as the antibiotic is absorbed across the peritoneal membrane. The length of the dwell period is an important factor as it determines the amount of antibiotic being absorbed through the peritoneal membrane. During active peritonitis when the peritoneal membrane is inflamed, enhanced absorption of aminoglycosides, vancomycin and cefazolin across the membrane can occur. (Low, Gopalakrishna et al. 2000) Following antibiotic absorption and

distribution into the vascular space, there is a much slower uptake of antibiotic back into the dialysate with subsequent exchanges of dianeal fluid.

Most intraperitoneal regimens produce sufficiently high antibiotic concentrations in the peritoneal cavity exceeding levels required to overcome resistant organisms. (Vas 1993; Low, Bailie et al. 1996; Lai, Kao et al. 1997; Low, Gopalakrishna et al. 2000) Cefazolin has a relatively low molecular weight and is transferred readily across the peritoneal membrane. During peritonitis the bioavailability of cefazolin is increased and cefazolin is rapidly absorbed systemically. (Low, Gopalakrishna et al. 2000) With intraperitoneal administration of cefazolin 70 to 80% of the dose is absorbed systemically. (Bunke, Aronoff et al. 1983; Low, Gopalakrishna et al. 2000) Regimens of intraperitoneal cefazolin (500mg to 1.5g/ 2L) once daily achieve intraperitoneal levels at 24h that are well above MIC values for many organisms. (Bunke, Aronoff et al. 1983; Manley, Bailie et al. 1999; Low, Gopalakrishna et al. 2000) The intraperitoneal level of cefazolin achieved with once daily dosing has been shown to be effective for treatment of even resistant *S.epidermidis* organisms. (Keane, Alexander et al. 1996)

Once daily dosing of aminoglycosides (0.6 to 2mg/kg) also achieves adequate trough levels in both serum and dialysate sufficient for appropriate peritonitis treatment while at the same time minimizing aminoglycoside toxicity. (Walshe, Morse et al. 1986; Tosukhowong, Eiam-Ong et al. 2001) Although treatment failure rates for pseudomonal peritonitis remain relatively high, treatment with intraperitoneal ceftazidime dosed at either 15mg/kg once daily, 1g/2L or 125mg/L with each exchange all produced dialysate

and serum concentration well above the MIC for most Gram-negative organisms and Gram-positive organisms. (Ryckelynck, Vergnaud et al. 1986; Demotes-Mainard, Vincon et al. 1993; Grabe, Bailie et al. 1999) Unlike most intraperitoneal administered antibiotics, which demonstrate enhanced absorption with inflammation of the peritoneal membrane, there was no increase in ceftazidime absorption across the peritoneal membrane in cases of active peritonitis. (Ryckelynck, Vergnaud et al. 1986)

High doses of vancomycin are administered once weekly to overcome limited peritoneal absorption. With intraperitoneal administration of vancomycin approximately 50 to 60% of the total intraperitoneal dose is absorbed across the peritoneal membrane.(Morse, Farolino et al. 1987) Following absorption there is relatively slow re-absorption of vancomycin back into the dialysate with subsequent exchanges throughout the week. Vancomycin has a slow distribution phase and the mean half-life following intraperitoneal administration is approximately 92h, this allows dialysate levels over a one-week period to remain >2 ug/ml. (Morse, Farolino et al. 1987)

2. Combination treatment

In the treatment of CAPD peritonitis both empiric treatment and treatment of *Pseudomonas* infections involves the use of combination antibiotics. Empiric therapy with combinations of cefazolin plus aminoglycoside or ceftazidime is initiated in all cases. Cephalosporins have activity against both Gram-positive and Gram-negative organisms and when used in combination with aminoglycosides have demonstrated

synergistic activity against *staphylococci* and *streptococci* species. (Keane, Alexander et al. 1996) A study comparing empiric treatment with intraperitoneal cefazolin plus tobramycin and vancomycin plus tobramycin against S.epidermidis peritonitis concluded that tobramycin therapy of less than two days was strongly associated with treatment failure. (Ariano, Franczuk et al. 2002)

3. Antibiotic activity in Dianeal fluid

Inhibitory effects on antibiotic activity have been demonstrated in both fresh and spent dianeal fluid collected from patients with and without peritonitis. It has been postulated that antagonisms of antibiotic activity by dianeal fluid may be a cause of treatment failure. Comparing MICs in dianeal fluid to broth is a common method of assessing antibacterial activity in dianeal fluid. Ciprofloxacin has been shown to be significantly less active in dialysate by exhibiting a 4-fold increase in MIC in dialysate. (Ludlam, Johnston et al. 1992) Reductions in *S.epidermidis* susceptibility to ciprofloxacin, imipenem and gentamicin have also been reported in dianeal fluid. (Wilcox, Edwards et al. 1985; Ludlam, Johnston et al. 1992) A study looking at changes in MICs and MBCs in dianeal fluid compared to broth also reported two-fold increases in MICs for cefazolin and vancomycin against *S.epidermidis.* (Zelenitsky, Franczuk et al. 2002)

When assessing the activity of gentamicin and ceftazidime against *P.aeruginosa* there was also a significant decrease in activity observed when compared to

broth. (Craddock, Edwards et al. 1987) Inhibition in bactericidal activity against *P. aeruginosa* has also been demonstrated for tobramycin, ciprofloxacin, ceftazidime and piperacillin. MBC levels measured in dianeal fluid were eight to 16 times greater than those measured in broth. (Shalit, Welch et al. 1985) A two to three fold increase in MICs was observed with ciprofloxacin and tobramycin in dianeal fluid for *P.aeruginosa.* Tolerance defined as a MIC:MBC ratio greater than 32 was observed with ceftazidime and piperacillin against *P.aeruginosa* in all dianeal fluids tested.(Zelenitsky, Franczuk et al. 2002)

3. LIMITATIONS OF ROUTINE MIC TESTING IN THE TREATMENT OF CAPD PERITONITIS

There is limited data available on the pharmacodynamic activity of intraperitoneal antibiotic administration. The current standard of MIC testing does not represent antibacterial activity within the peritoneal cavity. MICs are used in the treatment of peritonitis to provide information on antibiotic sensitivities of particular pathogens. However, MIC breakpoints are based on achievable antibiotic serum levels and corresponding activity required for effective treatment. MICs were not intended to represent activity in dianeal fluid and furthermore various studies have reported changes in MICs preformed in dianeal fluid. In the treatment of peritonitis effective antibacterial activity within the peritoneal cavity is necessary for treatment and the use of MIC data for antibiotic selection fails to provide information on the true pharmacodynamic activity at the site of infection.

Using broth as a growth medium for MIC testing produces optimal conditions required for growth and characteristic behaviors of the organisms. Differences in growth characteristics and environment have been shown to affect antibacterial activity in various types of dianeal fluid. This has been exhibited by changes in MICs and MBCs and static time kill preformed in dianeal fluid as compared to broth. MICs and MBCs are a measure of antibacterial activity in a protein free medium at an adjusted pH of 7.2 to 7.4. In the peritoneal cavity, factors such as proteins, pH, antibiotic bioavailability and electrolytes may affect antibiotic pharmacodynamics. Differences in antibiotic activity in dianeal fluid can be explained in some cases by changes in pH. Increases in MBCs have been observed for ciprofloxacin and tobramycin with *P.aeruginosa* in fresh dianeal fluid (pH 5.5) but not in buffered dianeal fluid (pH7.4). (Shalit, Welch et al. 1985) The biochemical composition of dianeal fluid is also subjected to inter-patient variability. Cation levels in broth have been shown to alter cellular uptake and antibacterial effect of aminoglycosides. (NCCLS 2000) In MIC testing the level of cations in broth, specifically Ca^{+2} and Mg^{+2} are adjusted. Routine MIC testing would not detect changes in antibiotic activity potentially associated with the biochemical composition of the dianeal fluid.

Tolerance observed in dianeal fluid especially with *P.aeruginosa*, may further illustrate the disadvantages of routine MIC testing. This is further exemplified by high pseudomonal peritonitis failure rates despite treatment with combinations of sensitive antibiotics. Although MIC values measured in broth are somewhat similar to those in

dianeal fluid, decreases in bactericidal activity against *Pseudomonas* have been observed. Even though the ability to inhibit bacterial growth remains relatively similar in dianeal fluid the ability to kill bacteria is impaired. Routine MIC testing in the treatment of peritonitis would not detect tolerant pathogens. Organisms reporting sensitive MIC breakpoints with no bactericidal activity in dianeal fluid would remain undetected potentially resulting in inadequate treatment.

There is limited clinical support for the use of MICs in the treatment of CAPD peritonitis. MIC sensitivity data is most commonly used in antibiotic selection and sensitivities obtained are often poorly associated with clinical response. A study by Ludlam et al. 1992 which examined bacterial isolates from 34 patient episodes of peritonitis treated with ciprofloxacin monotherapy concluded that although ciprofloxacin was significantly less active in dialysate there was no evidence to suggest that antagonism of antibiotic activity by dialysate lead of treatment failure. However, Craddock et al. 1987 investigated sources of clinical failure in five cases of pseudomonal peritonitis. They reported that reduced ceftazidime and gentamicin activity in dianeal fluid along with bacterial adherence to catheters were important factors in the clinical failure in all five cases. Further clinical indications that MICs may not correlate to clinical failure was shown in a study by Ariano et al. 2002 that concluded that treatment with intraperitoneal cefazolin and tobramycin was as effective as vancomycin and tobramycin for *S. epidermidis* peritonitis despite a high prevalence of methicillin resistance. This study also concluded that tobramycin therapy of less than two days was strongly associated with treatment failure. MIC testing is a measure of single antibiotic

activity and cannot assess the overall activity of antibiotic combinations. Since the majority of peritonitis episodes are treated with combinations of antibiotics, the beneficial effect of combination therapy such as the addition of tobramycin in *S. epidermidis* peritonitis treatment may be missed.

SUMMARY

In the treatment of CAPD related peritonitis, treatment failure rates can exceed 30%. (Piraino, Bernardini et al. 1987; Goldberg, Clemenger et al. 2001) In cases of pseudomonal peritonitis failure rates can be as high as 50%. (Piraino, Bernardini et al. 1987; Szeto 2001) The clinical implications of treatment failure such a catheter removal and transfer to hemodialysis can result in huge lifestyle changes for patients on CAPD. Inter-patient variability in dianeal fluid composition can potentially have an impact on pharmacodynamic activity within the peritoneal cavity. Studies have demonstrated that variability in dianeal fluid biochemistry; pH and degree of immune factor antagonism can all affect antibacterial activity. Inter-patient variability in peritoneal membrane permeability and its affect of antibiotic concentrations in dialysate can also alter the overall antibacterial activity within the peritoneal cavity. Treatment selection is currently based on MIC results, which have many limitations. MICs are preformed in broth at an adjusted pH and measure individual antibiotic sensitivities. However in addition to the lack of clinical support for their use in the treatment of peritonitis, MICs do not account for changes in antibiotic activity, nor are they capable of detecting loss of bactericidal activity in dianeal fluid. For an indication where antibiotic combinations are frequently

used, MICs are of limited benefit. In the relatively small CAPD population, there is limited data available comparing various treatment regimens because of challenges in recruiting patients for clinical studies. The development of a new microbiological test capable of measuring overall pharmacodynamic activity of combination therapy in dianeal fluid would contribute significantly to individual treatment selection and clinical guidelines for CAPD peritonitis.

PURPOSE AND OBJECTIVES

A. PURPOSE

To develop and evaluate a new microbiologic test, the PBT, which measures antibiotic activity in dianeal fluid. The study will examine the use of the PBT to compare treatment regimens, and its potential association with clinical outcome.

B. OBJECTIVES

1. DEVELOPMENT OF THE PBT TEST.

The first phase will involve development of the PBT test including a procedure for processing and storing spent dianeal fluid. Phase one will evaluate the PBT test for reproducibility against an array of the most common pathogens. It will also assess the effects of different dianeal fluids on PBT results.

2. CHARACTERIZATION OF THE PBT TEST.

Phase two will characterize the range of PBTs for the most commonly isolated organisms causing CAPD related peritonitis. PBTs will be preformed using a single sample of spent dianeal fluid and of two treatment regimens: (1) empiric IP cefazolin plus tobramycin once daily, and (2) IP cefazolin once daily, both at simulated 6 h and 24 h

concentrations. The PBT test will be used to compare the two regimens and two simulated concentration profiles.

3. PBT ASSOCIATION WITH CLINICAL OUTCOME.

In phase three, a pilot study will evaluate the association of PBTs with clinical outcome in 14 patients diagnosed with CAPD related peritonitis. PBTs will be measured using spent dianeal fluid collected prior to antibiotic therapy, simulated 6 h and 24 h concentrations of the antibiotics used to treat their infection, and the infecting organism. The PBT will then be tested for an association with clinical outcome.

C. GENERAL HYPOTHESIS

1. The PBT test will be an accurate and reproducible measurement of antibacterial activity in dianeal fluid.
2. The PBT will correlate to some extent with MIC and MBC data.
3. The PBT will measure differences in antibacterial activity between different dianeal fluids.
4. The PBT will correlate more closely to clinical outcome than traditional tests, MIC and MBC.

D. LIMITATIONS

The PBT test can only be applied to organisms that growth sufficiently in spent dianeal fluid. The PBT test can only be done with spent dianeal fluid from which contaminating organisms can be removed.

E. ASSUMPTIONS

1. The antibiotic concentrations achieved during the treatment of peritonitis in the patient population can be simulated.
2. The sensitivity profiles of the six organisms studied are representative of the pathogens associated with CAPD peritonitis in the patient population.

METHODS

A. DIANEAL FLUID

1. COLLECTION, PROCESSING AND STORAGE

Bags of spent dianeal fluid were collected from the Microbiology Laboratory after storage at 4°C for a maximum of one week or until culture results were obtained. All culture negative dianeal fluids were discarded. Only culture positive fluid collected from CAPD patients prior to antibiotic treatment was used.

Prior to sampling, the counter top and dianeal fluid bag were wiped with Hydrox® solution and gloves were worn. Bags of spent dianeal fluid were mixed well by shaking each bag until all cellular debris was dispersed. The dianeal fluid bag tubing was swabbed with alcohol and by releasing the tubing clamp; 45ml aliquots were poured into 50ml sterile centrifuge tubes. A separate 10ml aliquot was also collected and processed in a 15ml sterile centrifuge tube for biochemical analysis.

All samples of dianeal fluid were stored in sterile 50ml centrifuge tubes at –70°C for a maximum of 12 months. Prior to PBT testing 45ml dianeal fluid samples were removed from the –70°C freezer and slowly thawed to room temperature. Samples of completely thawed dianeal fluid were centrifuged at 3500rpm for 10minutes. Using a sterile 10ml pipette approximately 35ml of the supernatant was transferred into a new sterile 50ml centrifuge tube and re-centrifuged at 3500rpm for10minutues. Following the second centrifugation a new sterile 10ml pipette was used to transfer approximately 25 ml of the supernatant into a sterile 50ml centrifuge tube.

Collected dialysate bags were linked with a microbiology culture number and biochemistry sample number so that each frozen aliquot could be linked with culture results and the original bacterial isolate.

2. BIOCHEMICAL ANALYSIS

Biochemical analysis was preformed prior to and post storage at –70°C. Centrifugation was required prior to biochemical analysis; therefore a 10ml aliquot of dianeal fluid was centrifuged at 3500rpm for 10 minutes (x 2). Two ml of centrifuged dianeal fluid were sent to the Biochemistry Laboratory for protein, sodium, potassium, chloride, bicarbonate, glucose, urea, creatinine, calcium, phosphate and magnesium levels. pH measurements were preformed using ColorpHast® pH6.5-10.0 pH indicator strips.

3. QUALITY CONTROL

As a negative control for contaminant growth, 1 mL of processed spent dianeal fluid and 1 mL of M-H broth and one ml of M-H broth alone were incubated at 35°C for 48 h. Following incubation for 24 and 48h, 100ul samples of each were plated on TSA plates.

B. BACTERIA

1. SELECTION AND STORAGE

The most common CAPD peritonitis pathogens during the period of January 1999 to December 2001 were selected. A total of 36 strains, six of *S. aureus, S. epidermidis, E. coli, E. cloacae, K. pneumoniae* and *P. aeruginosa* previously isolated from patients with CAPD-related peritonitis were randomly selected. The majority of the isolates were collected during the year of 2001 except for *P.aeruginosa*, which occurred less commonly, and required isolates from January 1999. *P.aeruginosa,* although less common, was studied due to severity of infection and high treatment failure rates seen with this organism. α-Hemolytic streptococcus a commonly isolated organism in CAPD peritonitis could not be studied due to its poor growth in dianeal fluid. PBT trials using both 90% dianeal fluid and 50% dianeal fluid with M-H broth did not support growth of six different α-Hemolytic streptococcus isolates incubated at 35°C over 24 h.

Isolates were stored in skim milk at –70°C. Isolates were obtained directly from –70°C freezer stocks and sub cultured three times on TSA plates prior to PBT testing. Plates were stored at 2 to 8°C and sub cultured weekly. Every four weeks new isolates were obtained from the –70°C freezer culture stocks. Isolates were plated on TSA plates 24 h prior to each experiment.

2. GROWTH STUDIES IN DIANEAL FLUID

Growth studies were preformed to determine organism growth in dianeal fluid. Each isolate was tested in two samples of 50% dianeal fluid and one sample of 100% M-H broth. Two isolates each of *S.epidermidis, S. aureus, E.cloacae*, and *K.pneumoniae* were studied. Only one isolate each of *E.coli* and *P.aeruginosa* were available in stocked isolates during this time period.

Two bags of culture positive dianeal fluid were also obtained from the microbiology lab. Both bags were processed by centrifugation so as to remove contaminating organisms. One ml of each dianeal fluid and one ml of M-H broth were added to each test tube. A 0.5 McFarland turbidity standard was used to standardized inoculum density that would produce colony counts of 1 to 2 $x10^8$ CFU/mL. A direct suspension was prepared for each isolate by selecting isolated colonies from a 24h TSA plate and suspending the colonies in M-H broth. The suspension was adjusted to match a 0.5 McFarland standard. Within 15 minutes, the inoculum suspension was diluted 1: 100 in M-H broth to produce a required volume of a 1 x 10^6 CFU/mL suspension. One ml of the new suspension was used to inoculate each test tube (one ml M-H broth, one ml Dianeal fluid #1 and one ml Dianeal fluid #2) to produce a final volume of 2ml in each tube, which contained 5 x 10^5 CFU/mL. Colony counts were preformed immediately on the 1 x 10^6 CFU/ml suspension by serially diluting samples in cold sterile saline (0.9%) and plating 100ul of the 10^{-2},10^{-3} and 10^{-4} dilutions in duplicate on TSA plates. Colony counts between 10 and 100 were recorded. All tubes were vortexed and incubated at 35°C for 24h. Colony counts were

preformed the following day on all tubes by serially diluting samples in cold sterile saline (0.9%) and plating 10ul of the 10^{-6},10^{-7} and 10^{-8} dilutions in duplicate on TSA plates.

C. ANTIBIOTICS

1. STOCK SOLUTIONS

Cefazolin (Sigma 500mg Lot 20K0846), vancomycin (Sigma 500mg Lot 121K1140) and tobramycin (Sigma 500mg Lot 78H0452) were prepared in concentrations of 1000ug/ml. Sterile distilled water was used as the diluent for cefazolin, vancomycin and tobramycin. Cefazolin powder had a potency of 986ug/mg. To make a cefazolin stock solution (1000ug/mL), 100mg of cefazolin was weighed on an analytical balance and diluted in 98.6ml of sterile distilled water. Tobramycin powder had a potency of 648ug/mg. To make the tobramycin stock solution (1000ug/mL), 100mg of tobramycin was weighed on an analytical balance and diluted in 64.8ml of sterile distilled water.

Ceftazidime powder (Eli Lilly 1g) had a potency of 848ug/mg. To make a 1000 ug/ml solution, ceftazidime was solubilized according to NCCLS guidelines in anhydrous sodium carbonate at a weight of exactly 10% of the ceftazidime to be used. The sodium carbonate was solubilized in sterile distilled water and ceftazidime was dissolved in the sodium carbonate solution. Sterile distilled water was added until the desired volume was obtained. The ceftazidime solution could be stored for up to 6 h at 25°C.

Antibiotic stock solutions were adjusted based on the following equation.

Volume (ml)= actual weight (mg) X potency (ug/mL) / desired concentration (ug/mL)

2. STORAGE

All antibiotic powders were stored in a desiccator at 2°C to 8°C. Antibiotic stock solutions were stored in 1.8ml aliquots in 2mL size capped cryogenic tubes. According to NCCLS guidelines, stock solutions were stored at –70°C for a maximum duration of 6 months without any loss of activity. Aliquots were thawed to room temperature as needed and used the same day.

3. QUALITY CONTROL

Quality control MIC tests were preformed in duplicate on each new batch of cefazolin and tobramycin stock solutions. *S.aureus* ATCC 29213 was used as a control strain for both cefazolin and tobramycin stock solutions. *P.aeruginosa* ATCC 27853 was also used as a control strain for tobramycin stock solutions. Control strains were stored in 2ml size capped cryogenic tubes containing skim milk and stored at –70°C. Frozen strains were subcultured three times prior to testing. Acceptable quality control limits for MIC testing of *S.aureus* ATCC 29213 against cefazolin and tobramycin stock solutions were 0.25-1 ug/ml and 0.12-1 ug/ml respectively. Acceptable quality control limits for MIC testing of *P. aeruginosa* ATCC 27853 against tobramycin stock solution

were 0.25-1ug/ml. All antibiotic stock solutions underwent quality control MIC testing. All antibiotic stock solutions with MICs within the acceptable range were stored at –70^{O}C.

D. MIC AND MBC

MIC and MBC testing was performed by macro dilution in duplicate for all CAPD peritonitis isolates for cefazolin, tobramycin, vancomycin and ceftazidime. MIC and MBC testing was preformed according to NCCLS guidelines.(NCCLS 2000)

1. BROTH PREPARATION

Cation adjusted M-H broth at a pH of 7.2 – 7.4 was used in all MIC and MBC testing. MICs of aminoglycosides for *P.aeruginosa* are affected by the presence of divalent cations therefore cation adjusted M-H broth was recommended according to NCCLS guidelines. M-H Broth was prepared according to manufacturers directions by dissolving weighed amounts of broth powder into corresponding volumes of filtered water. All broth was autoclaved within the same day of preparation. Ca^{++} and Mg^{++} were added to the sterile broth to achieve concentrations of 20-25mg of Ca^{++}/L and 10-12.5mg of Mg^{++}/L. A 10mg of Mg^{++}/ml magnesium stock solution was prepared by dissolving 8.36g of $MgCl_2$*$6H_20$ in 100ml of sterile distilled water. A 10mg of Ca^{++}/mL calcium stock solution was prepare by dissolving 3.68 g of $CaCl_2$*$2H_2O$ in 100ml of sterile distilled water. Stock solutions were sterilized by membrane filtration and stored

at 2 to 8^0C.. To each liter of M-H broth, one ml of Mg^{++} stock solution and 2ml of Ca^{++} stock solution were added. All supplemented M-H broth was stored at 35^0C in clear glass bottles. Prior to use, all bottled broth were checked for contamination/ cloudiness and plated.

2. ANTIBIOTIC PREPARATION

Starting antibiotic concentrations were selected for each organism based on predicted MIC values. The starting antibiotic concentration present in the first tube determined the range of concentrations present in the subsequent 7 tubes. Ideally the MIC should be found in the middle of the 8-tube concentration range. Antibiotic stock solutions stored at –70°C were thawed and used the same day. Starting antibiotic concentration standards (working standards) were prepared by adding antibiotic stock solution to supplemented M-H broth to achieve the desired concentration. The concentration of the antibiotic working standard was double the required starting concentration because of a 1:2 dilution that occurs when an equal volume of the inoculum was added.

3. INOCULUM PREPARATION

A 0.5 McFarland turbidity standard was used to standardize inoculum density that would produce colony counts of 1 to 2 $x10^8$ CFU/mL. A direct suspension was prepared by selecting isolated colonies from a 24h TSA plate and suspending the colonies in

supplemented M-H broth. The suspension was adjusted to match a 0.5 McFarland standard. Within 15 minutes, the inoculum suspension was diluted 1: 100 in supplemented M-H broth to produce a required volume of a 1 x 10^6 CFU/mL suspension. Colony counts were preformed immediately on the 1 x 10^6 CFU/ml suspension by serially diluting samples in cold sterile saline (0.9%) and by plating 100ul of the 10^{-2},10^{-3} and 10^{-4} dilutions in duplicate on TSA plates.

4. MIC/ MBC DETERMINATIONS

All test were preformed in sterile 13 x 100mm test tubes with plastic caps. Each test row contained eight test tubes to allow for eight serial dilutions of the antibiotic. One ml of supplemented M-H broth was added to tubes two through eight. A 2ml aliquot of the antibiotic working standard was added to the first tube. The serial transfer of one ml starting from the first tube, with one ml being discarded from the eighth tube produced a final volume of 1 ml in each tube. One ml of the inoculum suspension was used to inoculate each test tube to produce a final volume of 2ml in each tube, which contained 5 x 10^5 CFU/mL. All tubes were vortexed and incubated at 35°C for 18-24 h. The MIC was determined by visual inspection of each vortexed tube as the lowest concentration that inhibited organism growth. (i.e., first visibly clear tube) MBCs were determined by plating 10ul from each clear tube in duplicate on TSA plates. MBCs were determined as the lowest concentration to produce a 3-log kill (99.9% kill) from the initial inoculum count.

5. QUALITY CONTROL

Positive controls were used for each organism and consisted of 1 ml of M-H broth and 1 ml of $1x10^6$ CFU/ml inoculum suspension. The MIC was considered valid if the positive control tube was turbid following a 24-h incubation at 35^0C. Positive controls were also used as purity controls and plated in duplicate on TSA plates and incubated overnight at 35^0C to detect mixed cultures.

Negative controls consisted of 1 ml of M-H broth. The MIC was considered valid if the negative control tube was clear following a 24-h incubation at 35^0C.

E. PERITONEAL BACTERICIDAL TITRE

The PBT measures overall antibacterial effect including antibiotic combinations in dianeal fluids. Samples of dianeal fluid during antibiotic therapy are serially diluted and inoculated with the infecting pathogen. The PBT is the maximum dilution, which maintains bactericidal activity. It measures the intrinsic antibacterial effect including antibiotics and other contributing factors in the dianeal fluid.

PHASE 1: PBT Development

The effect of dianeal fluid on PBTs was evaluated. PBTs for six organisms were measured in six dianeal fluid samples at predicted 6h and 24h

dialysate antibiotic concentrations determined from the literature, for the empiric regimen of cefazolin and tobramycin.

PHASE 2: Characterization of PBT

PBTs for six isolates each of six organisms (total of 36 isolates) were measured in one dianeal fluid at predicted 6h and 24h dialysate antibiotic concentrations for the empiric regimen of cefazolin and tobramycin and for treatment with cefazolin alone.

PHASE 3: Pilot Study

PBTs were measured in dianeal fluid collected from patients with peritonitis at predicted 6h and 24h dialysate concentration of the patient's own antibiotic treatment. PBTs were related to clinical outcome.

1. PBT DETERMINATION

1. Antibiotic Preparation

Dianeal fluid was collected from patients with peritonitis who had not yet received antibiotic treatment. For the initial characterization of the PBT, the average range of intraperitoneal antibiotic concentrations obtained with the empiric dosing regimens was required. Inter-patient variability in antibiotic concentrations following a 6h dwell and 24h period would lead to highly variable PBTs for the same isolates.

Therefore in order to control this variable, antibiotic concentrations were predicted from the literature and antibiotics were added to the spent dianeal fluid. The combination of once daily intraperitoneal cefazolin and tobramycin is the current empiric regimen used at St. Boniface Hospital. Cefazolin and tobramycin were added to dianeal fluids to simulate predicted dialysate concentrations at 6 and 24 h following a single intraperitoneal dose of 1.5g of cefazolin and 60mg of tobramycin added to 2L of dialysate following a 6 h dwell period. For a Gram-positive organism, aminoglycoside therapy would be discontinued and cefazolin continued at the same dose. In the pilot study some patients were started on vancomycin 2g/ 2L every seven days and aminoglycoside once daily and others were treated with ceftazidime 125mg/L in each bag and aminoglycoside once daily. Predicted 6h and 24h dialysate concentrations of vancomycin and ceftazidime were also obtained from the literature.

Antibiotic stock solutions stored at –70°C were thawed and used the same day. Antibiotic concentration standards (working standards) were prepared by adding antibiotic stock solution to a required volume of processed dianeal fluid to replicate specific concentrations at 6 and 24h time points. Antibiotic concentrations at 6 h following a 6-h dwell were replicated by simulating concentrations of 116ug/ml for cefazolin and 20ug/ml for tobramycin in processed dianeal fluid. (Walshe, Morse et al. 1986; Low, Gopalakrishna et al. 2000) To replicate antibiotic concentration seen at 24-h following a 6-h dwell, antibiotics were added to the processed dianeal fluid in concentrations of 32ug/ml for cefazolin and 3.2ug/ml for tobramycin. (Walshe, Morse et al. 1986; Low, Gopalakrishna et al. 2000) Once weekly vancomycin intraperitoneal

administration of 30 mg/kg with 6h dwell periods will produce average dialysate concentrations of approximately 610 ug/ml following a 6h dwell and 5 ug/ml at 24h. (Morse, Farolino et al. 1987) Repeated administration of ceftazidime 125mg/L into each dialysate bag with each exchange results in an average dialysate concentration of 30ug/ml following the initial 6h dwell and 43ug/ml at 24h (i.e. end of the fourth dwell period). (Ryckelynck, Vergnaud et al. 1986)

Table 1: Six hour and 24h dialysate concentrations of intraperitoneal antibiotics.

	6h concentrations (ug/mL)	24h concentration (ug/mL)
Cefazolin 1.5g/ 2L once daily	116	32
Tobramycin 60mg/2L once daily	20	3.2
Ceftazidime 125mg/L in every bag	30	43
Vancomycin 2g/ 2L every 7 days	610	5

2. Inoculum Preparation

A 0.5 McFarland turbidity standard was used to standardized inoculum density that would produce colony counts of 1 to 2 x10^8 CFU/mL. A direct suspension was prepared by selecting isolated colonies from a 24h TSA plate and suspending the colonies in 2 mL of un supplemented M-H broth. The suspension was adjusted to visually match a 0.5 McFarland standard. Colony counts were preformed immediately on the 1 to 2 x 10^8CFU/ml suspension by serially diluting samples in cold sterile saline (0.9%) and by plating 100ul of the 10^{-4},10^{-5} and 10^{-6} dilutions in duplicate on TSA plates.

3. PBT Experiment

All tests were preformed in sterile 13 x 100mm test tubes with plastic caps. Each test row contained 8 test tubes to allow for eight serial dilutions of the dianeal fluid. One ml of un supplemented M-H broth was added to tubes two through 8. Un-supplemented M-H broth was used as a diluent in the PBT. This provided a medium of growth for all isolates studied with minimal alteration to the biochemical composition of the spent dianeal fluid. It is proposed that other factors other than antibiotic affect may alter the overall antibacterial activity within this fluid. Therefore each sample of dianeal fluid exhibits specific intrinsic antibacterial activity. When the dianeal fluid was serially diluted with un-supplemented M-H broth, the antibacterial components within the dianeal fluid as well as antibiotic concentrations were both serially diluted in equal proportions. Consequently, the overall antibacterial effect present within the initial tube was serially diluted and not simply the antibiotic alone.

Following the addition of broth to tubes two to eight, a 2ml aliquot of the working standard (dianeal fluid + antibiotics) was added to the first tube. The serial transfer of 1 ml starting from the first tube, with 1 ml being discarded from the eighth tube produced a final volume of 1 ml in each tube. Within 15 minutes of inoculum preparation, 10ul aliquots of the inoculum suspension were added directly below the fluid surface of each tube to yield a final inoculum of 1×10^6CFU/ml.. All tubes were incubated at 35°C for 18-24 h.

PBTs were initially determined by plating 10ul from each clear tube in duplicate on TSA plates. PBTs were defined as the lowest concentration to produce a 3-log kill (99.9% kill) from the initial inoculum count. (i.e. bactericidal kill) The first clear tube generally exhibited a bactericidal kill therefore for the later part of the PBT tests, the PBT was subsequently determined as the lowest concentration inhibiting visible bacterial growth.

4. Quality Control

Positive controls were used for each organism and consisted of 1 ml of un-supplemented M-H broth and 10ul of $1x10^8$ CFU/ml inoculum suspension. The PBT was considered valid if the positive control tube was turbid following a 24-h incubation at 35^0C. Positive controls were also used as purity controls and plated in duplicate on TSA plates and incubated overnight at 35^0C to detect mixed cultures.

Negative controls consisted of one ml of un-supplemented M-H broth and one ml of un-supplemented M-H broth with one ml of processed dianeal fluid. The PBTs were considered valid if the negative control tubes were clear following a 24-h incubation at 35^0C.

2. Pilot Study

Prior to collecting dianeal fluids, ethics approval was obtained. Patients were identified through the collection of dianeal fluid sent to the Microbiology lab for Gram stain and culture. Culture positive dianeal fluid and corresponding isolate were stored at -70^0C. As described previously, PBTs were preformed with processed dianeal fluid that was spiked with predicted antibiotic concentrations at 6h and 24h obtained from the literature. PBTs, MICs and MBCs were preformed for each isolate as described in the previous methodology.

Treatment and clinical outcome data was obtained from patient charts within the peritoneal dialysis clinic and cross-referenced with data obtained from the St. Boniface Hospital (Winnipeg, Canada) peritonitis database (Baxter POET ™ 2.1 Clinical Monitoring System). Treatment failure was defined as either a reoccurring infection within 14 days, catheter removal and transfer to hemodialysis, death or change in therapy excluding changes due to allergy

F. STATISTICAL ANALYSIS

Statistical analysis was performed using Microsoft Excel and SPSS software programs for Windows. The level of significance for all analysis was assessed at an alpha level of 0.05.

Paired two-tailed t-tests were used to examine differences in the biochemical composition between dianeal fluids. Median and interquartile range were used to

characterize PBTs for each organism. Scattergrams of PBTs versus antibiotic concentrations at 6 and 24 h divided by MBCs were used to examine relationships between PBTs and MBCs. The Mann-Whitney test was preformed along with scattergrams of PBTs to examine relationships between cefazolin and tobramycin and cefazolin alone regimens at 6h and 24 h concentrations. The Mann-Whitney test was preformed and the exact significance value was reported in the PBT analysis and clinical outcome.

RESULTS

A. DIANEAL FLUID

1. COLLECTION AND PROCESSING

Centrifugation was sufficient in eliminating contaminant organisms from culture positive dianeal fluid bags. Of the 33 culture positive dianeal fluid bags collected only one dianeal fluid bag positive for *Stenotrophomonas maltophila,* when incubated with 50% M-H broth and plated, showed organism growth following the centrifugation procedure. A third centrifugation of the dianeal fluid from this same bag at 3500rpm for 10 minutes also failed to remove the organism.

Processed dianeal fluid and dianeal fluid diluted with 10% M-H broth incubated at 35^0C and at room temperature for 24 h produced a cloudy solution with an oily precipitate present on the fluid surface and walls of the test tube as shown in Table 2. Further investigation was required to verify that contamination was not the cause of the cloudy precipitate and to investigate the range dianeal fluid dilutions that would not form a precipitate. Colony counts on this precipitate were negative. Further studies were preformed at various temperatures, antibiotic combinations and broth dilutions to examine the conditions of precipitate formation. Processed dianeal fluid refrigerated at 8^0C and dianeal fluid diluted with 50% M-H broth incubated at 35^0C and at room temperature did not produce a precipitate. Processed dianeal fluid and dianeal fluid with 10% M-H broth containing 6h or 24h concentrations of cefazolin or cefazolin and

tobramycin with or without the organism added incubated at 35^{0}C for 24 h produced a cloudy solution with an oily precipitate present on the surface of the dianeal fluid and walls of the test tube. Colony counts on this precipitate were negative except for tubes that were spiked with organisms that demonstrated no inhibition to 6h or 24h cefazolin or cefazolin plus tobramycin concentration. Subsequent testing using 90% processed dianeal fluid and 10% Mueller-Hinton broth with organisms added produced the same oily precipitate following incubation at 35^{0}C for 24 h and colony counts on this solution also detected growth. Processed dianeal fluid diluted with 50% M-H broth containing 6h and 24-h concentrations of cefazolin or cefazolin and tobramycin with or without added organisms did not produce a precipitate following incubation at 35^{0}C and at room temperature for 24 h. Therefore in all PBTs the first tube containing 100% processed dianeal fluid, antibiotic and organism could not be evaluated by visual cloudiness and required plating in order to detect organism growth.

Table 2: Evaluation of precipitate formed in two samples of processed dianeal fluid at 6h and 24 h concentrations of cefazolin and tobramycin with *E.coli* and *S.epidermidis*.

Dianeal Fluid	*E.coli*		*S.epidermidis*	
	Precipitate	CFU/mL	Precipitate	CFU/mL
100% Dianeal Fluid at 35C	Cloudy/ oily	0	Cloudy/ oily	0
100% Dianeal Fluid at room temperature	Cloudy/ oily	0	Cloudy/ oily	0
100% Dianeal Fluid refrigerated	No precipitate	0	No precipitate	0
90% Dianeal Fluid at 35 C	Cloudy/ oily	0	Cloudy/ oily	0
90% Dianeal Fluid at room temperature	Cloudy/ oily	0	Cloudy/ oily	0
90% Dianeal Fluid refrigerated	No precipitate	0	No precipitate	0
50% Dianeal Fluid at 35 C	No precipitate	0	No precipitate	0
50% Dianeal Fluid at room temperature	No precipitate	0	No precipitate	0
50% Dianeal Fluid refrigerated	No precipitate	0	No precipitate	0
100% Dianeal Fluid + antibiotic at 35C	Cloudy/ oily	0	Cloudy/ oily	0
100% Dianeal Fluid + antibiotic at room temperature	Cloudy/ oily	0	Cloudy/ oily	0
100% Dianeal Fluid + antibiotic refrigerated	No precipitate	0	No precipitate	0
90% Dianeal Fluid + antibiotic at 35 C	Cloudy/ oily	0	Cloudy/ oily	0
90% Dianeal Fluid + antibiotic at room temperature	Cloudy/ oily	0	Cloudy/ oily	0
90% Dianeal Fluid + antibiotic refrigerated	No precipitate	0	No precipitate	0
50% Dianeal Fluid + antibiotic at 35 C	No precipitate	0	No precipitate	0
50% Dianeal Fluid + antibiotic at room temperature	No precipitate	0	No precipitate	0
50% Dianeal Fluid + antibiotic refrigerated	No precipitate	0	No precipitate	0
100% Dianeal Fluid + organism at 35C	Cloudy/ oily	$>1 \times 10^8$	Cloudy/ oily	$>1 \times 10^8$
100% Dianeal Fluid + organism at room temperature	Cloudy/ oily	$>1 \times 10^8$	Cloudy/ oily	$>1 \times 10^8$
90% Dianeal Fluid + organism at 35 C	Cloudy/ oily	$>1 \times 10^8$	Cloudy/ oily	$>1 \times 10$
90% Dianeal Fluid + organism at room temperature	Cloudy/ oily	$>1 \times 10^8$	Cloudy/ oily	$>1 \times 10^8$
50% Dianeal Fluid + organism at 35 C	Cloudy	$>1 \times 10^8$	Cloudy	$>1 \times 10^8$
50% Dianeal Fluid + organism at room temperature	Cloudy	$>1 \times 10^8$	Cloudy	$>1 \times 10^8$
100% Dianeal Fluid + organism + antibiotic at 35C	Cloudy/ oily	0	Cloudy/ oily	0
100% Dianeal Fluid + organism + antibiotic at room temperature	Cloudy/ oily	0	Cloudy/ oily	0
90% Dianeal Fluid + organism + antibiotic at 35 C	Cloudy/ oily	0	Cloudy/ oily	0
90% Dianeal Fluid + organism + antibiotic at room temperature	Cloudy/ oily	0	Cloudy/ oily	0
50% Dianeal Fluid + organism + antibiotic at 35 C	No precipitate	0	No precipitate	0
50% Dianeal Fluid + organism + antibiotic at room temperature	No precipitate	0	No precipitate	0

2. BIOCHEMICAL ANALYSIS

Table 3 shows the biochemistry and pH of spent dianeal fluid prior to freezing. The least amount of varibility between collected dianeal fluids was seen with Ca (1.36± 0.12mmol/L) electrolytes. The greatest inter-patient variability was seen in protein (1.314 ± 0.799 g/L) and glucose (24.3 ± 14.5 mmol/L) levels. Small differences in Na (128±23 mmol/L), Cl (93 ± 21 mmol/L), K (3.4 ± 0.6mmol/L), HCO_3 (22.5 ± 4.0 mmol/L), urea (15.1 ± 4.1 mmol/L) and Mg (0.65 ± 0.16 mmol/L) were measured with slightly more inter-patient variability seen with creatinine (616 ± 255 umol/L) and PO_4 (1.20 ± 0.47) levels. The average pH of all dianeal fluid collected was 7.4 ± 0.4 with little variability between dianeal fluids. Storage at -70^0C for 6 months period had no effect on the biochemistry composition as shown in Table 4. Decreases in calcium and increases in magnesium and creatinine levels were observed following storage at -70^0C for 12 months as shown in Table 5.

Table 3: pH and biochemistry of processed spent dianeal fluid prior to freezing. (n=33)

Dianeal	pH	Na^+ (mmol/L)	K^+ (mmol/L)	Ca^{+2} (mmol/L)	Mg^{+2} (mmol/L)	Cl^- (mmol/L)	HCO_3^- (mmol/L)	PO_4^- (mmol/L)	Urea (mmol/L)	Creatinine (umol/L)	Protein (mg/L)	Glucose (mmol/L)
1	8.0	136	2.7	1.27	0.64	98.0	19.7	1.34	15.8	588	0.565	47.5
2	8.0	140	3.8	1.38	0.84	104.0	24.2	1.40	12.4	795	1.280	26.0
3	7.0	134	4.4	1.19	0.44	102.0	21.5	0.32	11.6	388	0.512	6.6
4	8.0	131	2.1	1.24	0.60	94.0	22.6	0.94	8.3	326	0.773	30.4
5	7.0	131	3.4	1.33	0.63	100.0	22.8	0.80	15.5	487	2.355	6.9
6	8.0	132	2.8	1.53	0.54	96.0	26.5	0.95	10.0	447	0.676	27.0
7	7.0	129	3.5	1.47	0.90	96.0	21.1	1.30	16.1	501	0.711	33.5
8	8.0	130	2.6	1.37	0.40	94.0	22.7	0.69	10.2	496	0.461	26.2
9	7.0	136	3.4	1.47	0.62	100.0	24.6	1.17	16.9	762	2.242	1.5
10	7.0	133	3.3	1.21	0.43	95.0	21.4	1.25	17.6	559	0.589	40.8
11	7.0	132	3.7	1.30	0.44	94.0	20.5	0.70	15.7	513	1.000	41.5
12	8.0	133	3.7	1.49	0.73	99.0	29.2	1.48	14.7	705	2.000	13.0
13	7.1	131	2.3	1.30	0.53	92.0	14.7	0.52	6.8	253	1.000	48.5
14	7.4	130	2.9	1.45	0.43	95.0	17.0	0.89	15.3	430	0.734	32.7
15	7.4	133	3.8	1.28	0.49	97.0	23.4	1.54	19.1	771	0.709	27.3
16	7.4	143	4.2	1.40	0.64	102.0	27.4	1.16	10.0	494	2.875	18.5
17	7.7	135	3.5	1.24	0.60	100.0	21.4	1.00	11.1	406	0.921	44.5
18	8.1	132	3.2	1.29	0.64	93.0	28.8	1.98	17.4	892	1.144	18.5
19	7.7	126	4.2	1.41	0.63	20.3	20.3	1.86	16.9	569	1.786	19.2
20	8.1	134	3.1	1.53	0.58	\	26.2	0.73	21.8	450	1.797	7.6
21	7.1	132	4.1	1.33	0.73	10.1	15.1	1.44	14.9	760	2.745	8.8
22	7.7	122	4.2	1.50	0.54	\	20.4	0.64	17.5	398	0.608	25.4
23	7.7	131	3.6	1.34	0.50	91.0	18.1	1.35	17.8	916	0.565	55.5
24	7.7	133	3.3	1.53	0.95	101.0	22.2	1.63	17.2	680	1.078	26.0
25	7.7	131	2.9	1.24	0.93	100.0	16.4	1.33	20.9	882	0.000	45.4
26	7.7	126	3.0	1.46	0.61	87.0	27.1	1.07	14.0	525	2.772	7.7
27	7.9	132	3.0	1.14	1.32	95.0	29.0	0.69	9.3	770	1.107	28.5
28	8.1	137	4.0	1.41	1.02	108.0	19.0	1.25	13.4	941	1.371	16.8
29	8.1	135	5.0	1.64	0.75	102.0	20.6	2.02	24.2	725	2.481	7.0
30	7.4	139	3.6	1.41	0.67	107	23.4	1.57	17.7	784	2.014	11.5
31	7.4	142	3.3	1.38	0.76	111	20.1	2.43	21.6	1523	2.304	8.8
32	7.8	136	4.1	1.30	0.86	105	27.9	0.88	12.7	349	1.613	21.3
33	7.8	125	2.9	1.20	0.67	92	25.7	0.73	13.2	257	0.584	21.3
Mean	7.35	128	3.4	1.36	0.65	93	22.5	1.20	15.1	616	1.314	24.3
sd	0.418	23	0.6	0.12	0.16	21	4.0	0.47	4.1	255	0.799	14.5

Table 4. Biochemical composition of 11 spent dianeal fluid samples prior to and following a six-month storage at –70°C. (Pre-storage value/ post-storage value)

Dianeal Fluid	Na (mmol/L)		K (mmol/L)		Ca (mmol/L)		Mg (mmol/L)		Cl (mmol/L)		HCO_3 (mmol/L)		PO_4 (mmol/L)		Urea (mmol/L)		Creatinine (umol/L)		Protein (mg/L)		Glucose (mmol/L)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
1	136	157	2.7	3.1	1.27	1.51	0.64	0.50	98.0	113.0	19.7	21.9	1.34	1.53	15.8	17.8	588	672	0.565	0.681	47.5	56.0
2	134	168	4.4	5.5	1.19	1.52	0.44	0.54	102.0	129.0	21.5	27.3	0.32	0.44	11.6	13.9	388	469	0.512	0.696	6.6	8.5
3	140	152	3.8	4.1	1.38	1.44	0.84	0.70	104.0	112.0	24.2	26.6	1.40	1.48	12.4	13.7	795	843	1.280	1.337	26.0	27.2
4	131	107	2.1	1.7	1.24	1.30	0.60	0.42	94.0	74.0	22.6	18.5	0.94	0.75	8.3	6.9	326	257	0.773	0.679	30.4	23.6
5	132	140	2.8	2.9	1.53	1.65	0.54	0.54	96.0	102.0	26.5	26.4	0.95	1.01	10.0	10.2	447	458	0.676	0.743	27.0	29.6
6	134	132	3.1	3.0	1.53	1.49	0.58	0.82	101.0	102.0	26.2	28.0	0.73	0.75	21.8	21.8	450	452	1.797	1.975	7.6	7.0
7	133	130	3.3	3.4	1.53	1.44	0.95	1.04	101.0	102.0	22.2	21.0	1.63	1.59	17.2	16.8	680	668	1/078	1.233	26.0	26.0
8	137	134	4.0	3.8	1.41	1.36	1.02	1.14	108.0	108.0	19.0	18.0	1.25	1.36	13.4	13.1	941	894	1.371	1.462	16.8	16.0
9	132	131	3.0	3.0	1.14	1.27	0.69	0.66	95.0	99.0	29.0	30.0	1.32	1.28	9.3	9.5	770	741	1.107	0.999	28.6	29.4
10	126	123	3.0	3.0	1.46	1.37	0.61	0.85	87.0	94.0	27.1	27.0	1.07	1.11	14.0	14.3	525	546	2.772	3.110	7.7	7.0
11	131	122	3.6	3.3	1.34	1.28	0.50	0.45	91.0	96.0	18.1	18.9	1.35	1.34	17.8	17.2	916	851	0.565	0.513	55.0	51.0
Mean	122	125	3.0	3.1	1.25	1.30	0.62	0.64	90.0	94.0	21.3	22.0	1.03	1.05	12.6	12.9	569	571	1.166	1.119	23.3	23.4
sd	4	17	0.7	0.9	0.14	0.12	0.19	0.24	6.1	13.7	3.6	4.4	0.37	0.37	4.1	4.2	214	203	0.713	0.765	15.7	16.4
P value	0.57		0.48		0.22		0.62		0.18		0.39		0.34		0.36		0.89		0.11		0.87	

Table 5. Biochemical composition of six dianeal fluid samples prior to and following a 12-month storage at –70°C.

Dianeal Fluid	Na (mmol/L)		K (mmol/L)		Ca (mmol/L)		Mg (mmol/L)		Cl (mmol/L)		HCO_3 (mmol/L)		PO_4 (mmol/L)		Urea (mmol/L)		Creatinine (umol/L)		Protein (mg/L)		Glucose (mmol/L)	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
1	131	135	3.4	3.5	1.33	1.32	0.63	0.81	100	104	22.8	22.4	0.8	0.8	15.5	16.7	487	525	2.355	2.354	6.9	6.8
2	133	132	3.3	3.3	1.21	1.19	0.43	1.09	95	94	21.4	20.9	1.25	1.25	17.6	18.5	559	587	0.589	0.664	40.8	37.5
3	133	131	3.7	3.7	1.49	1.42	0.73	0.94	99	101	29.2	28	1.48	1.61	14.7	14	705	722	2.000	1.630	13.0	12.2
4	130	122	2.9	2.8	1.45	1.39	0.43	0.68	95	91	17	16	0.89	0.88	15.3	15.2	430	437	0.734	0.720	32.7	32.0
5	126	127	4.2	4.1	1.41	1.29	0.63	0.98	93	93	20.3	21.4	1.86	1.6	16.9	17.4	569	588	1.786	1.807	19.2	18.4
6	122	120	4.2	4.1	1.5	1.45	0.54	0.61	-	-	20.4	21	0.64	0.66	17.5	18	398	401	0.608	0.641	25.4	23.7
Mean	129	128	3.6	3.6	1.40	1.34	0.57	0.85	96	97	21.9	21.6	1.2	1.1	16.3	16.6	525	543	1.345	1.303	23.0	21.8
sd	4	6	0.5	0.5	0.11	0.10	0.12	0.18	3	6	4.1	3.8	0.5	0.4	1.2	1.7	111	116	0.791	0.728	12.6	11.7
P value	0.45		0.36		0.02		0.02		0.89		0.56		0.72		0.23		0.02		0.55		0.04	

B. BACTERIA

Growth studies in Mueller-Hinton broth and two samples of dianeal fluid demonstrated similar growth for all six organisms over 24 h as shown in Table 6. All organisms grew well in 50% dianeal fluid (1 ml inoculum in M-H broth added to 1 ml of dianeal fluid) and therefore could be used in PBT testing. Three isolates of alpha-hemolytic streptococci were also tested, none of the isolates grew in 50% dianeal fluid and therefore could not be used in PBT testing.

Table 6: Growth Studies in 100% M-H broth versus 50% dianeal fluid and 50% M-H Broth following a 24-h incubation at 35^0C.

Organism	Isolate #	Initial Inoculum (CFU/mL)	24h count Dianeal Fluid #2 (CFU/mL)	24h count Dianeal Fluid#3 (CFU/mL)	24h count Mueller-Hinton Broth (CFU/mL)
S.epidermidis	25	1.0×10^6	$>1 \times 10^{10}$	1.1×10^9	$>1 \times 10^{10}$
	26	2.5×10^6	1.4×10^9	1.6×10^9	$>1 \times 10^{10}$
S.aureus	10	2.2×10^6	$>1 \times 10^{10}$	1.2×10^9	1.2×10^9
	11	2.0×10^6	1.6×10^9	$>1 \times 10^{10}$	$>1 \times 10^{10}$
E.coli	9	1.7×10^6	1.5×10^9	2.0×10^9	1.4×10^9
K.pneumoniae	2	1.1×10^6	1.1×10^9	1.2×10^9	3.7×10^9
	6	1.3×10^6	1.0×10^9	1.3×10^9	1.2×10^9
E.cloacae	1	1.9×10^6	1.6×10^9	1.6×10^9	1.8×10^9
	3	1.7×10^6	2.4×10^9	2.5×10^9	4.5×10^9
P.aeruginosa	30	6.0×10^6	2.2×10^9	1.8×10^9	1.3×10^9

C. MIC AND MBC

Table 7 shows MICs and MBCs for the 36 isolates. Of the six *S.epidermidis* isolates, four isolates were resistant to tobramycin with MICs ranging from 64 to >128 ug/ml. All *S.epidermidis, S.aureus, E.coli* and *K.pneumoniae* isolates were sensitive to cefazolin and tobramycin, with the exception of one *E.coli* isolate that was intermediately sensitive to cefazolin (MIC = 16 ug/ml) and resistant to tobramycin (MIC =64 ug/ml). All six *E.cloacae* isolates were resistant to cefazolin with MICs ranging from 128 to >256 ug/ml and sensitive to tobramycin. All six *P.aeruginosa* isolates were sensitive to tobramycin with MICs of 2 ug/ml or 4 ug/ml. MBCs for all isolates were either equal to or one to two fold greater than the MICs.

Table 7: MIC and MBC data for 36 CAPD peritonitis isolates

Organism	Isolate #	MIC (ug/ml)		MBC (ug/ml)	
		Cefazolin	**Tobramycin**	**Cefazolin**	**Tobramycin**
S.epidermidis	14	4	>128	8	>128
	18	0.25	0.5	4	2
	20	0.5	0.25	0.5	1
	23	2	64	4	128
	25	2	64	4	128
	26	1	64	2	128
S.aureus	4	1	2	4	2
	7	0.25	4	0.5	4
	10	1	2	8	4
	11	1	1	16	4
	21	0.5	2	0.5	4
	24	1	8	8	8
E.coli	12	2	4	4	4
	15	2	4	2	4
	16	4	4	4	4
	28	2	4	2	4
	30	16	64	32	64
	9	4	4	8	4
K.pneumoniae	2	4	2	4	2
	6	2	1	4	1
	13	4	2	4	2
	19	4	2	4	2
	31	4	1	4	2
	32	2	2	2	2
E.cloacae	1	>256	2	>256	2
	3	128	2	128	2
	22	>256	2	>256	2
	5	256	2	256	4
	29	>256	2	>256	2
	17	>256	2	>256	2
P.aeruginosa	37	>256	4	>256	4
	30	>256	4	>256	4
	33	>256	4	>256	4
	34	>256	2	>256	2
	35	>256	2	>256	4
	36	>256	2	>256	4

(Cefazolin MIC ≤ 8ug/ml = Sensitive, Cefazolin MIC 16 ug/ml = Intermediate, Cefazolin MIC ≥ 32ug/ml = Resistant, Tobramycin MIC ≤ 4 ug/ml =Sensitive, Tobramycin MIC= 8 ug/ml = Intermediate, Tobramycin MIC≥ 16 ug/ml = Resistant)

D. PERITONEAL BACTERICIDAL TITRE

PHASE 1: PBT DEVELOPMENT

1. MEASUREMENT AND REPRODUCIBILITY

PBT tests were preformed in duplicate and demonstrated a high degree of reproducibility. PBTs conducted by two individuals in two separate labs also exhibited reproducible titres. Of the 90 PBT tests preformed in duplicate, 69 tests (77%) had equivalent titres, 20 tests (22%) differed by +/- one dilution and only one test differed by a two-fold dilution and the test was repeated. The PBT results were therefore reported to an accuracy of +/- one dilution 99% of the time. Skipped tubes were occasionally seen, when this occurred the highest titre was read.

The first 72 PBT tests were measured both by visual inspection of the first clear tube (inhibitory titre) and by colony counts to determine a 3 log kill (bactericidal titre). Sixty-four of the 72 tests (89%) exhibited inhibitory and bactericidal counts that were equal or differed by +/- one dilution. Subsequently, PBTs were determined by visual inspection of the first clear test tube.

2. VARIABILITY WITH DIANEAL FLUIDS

Figure 1a & 1b show the PBTs at 6h and 24 h concentrations of cefazolin plus tobramycin PBTs for one isolate of each organism against six different dianeal fluids. Bearing in mind that the PBT test has an accuracy of +/- one dilution, significant differences were reported if PBTs differed by ≥ two-fold. PBTs at 6h showed no significant differences in all six dianeal fluids for all organisms, with the exception of *P.aeruginosa* PBTs which ranged from 1/8 to 1/32. At 24h concentrations, *E.cloacae, S.aureus, E.coli* and *S.epidermidis* exhibited greater variability in PBTs, which did not span more than two dilutions. Both *E.cloacae* and *E.coli* 24h PBTs ranged from ½ to 1/8 and 1/8 to 1/32 respectively. There also were similar differences in 24h PBTs for *S. aureus* and *S.epidermidis*, which ranged from 1/16 to 1/64 and 1/64 to 1/128 respectively.

Dianeal fluid collected from different patients demonstrated an affect on PBTs. Therefore there may be factors other than antibiotic activity that contribute to the overall antibacterial effect in dianeal fluid. To eliminate inter-patient variability in dianeal fluid a single dianeal fluid bag was used to evaluate and compare isolate sensitivities in the subsequent phase two studies.

Figure 1a: PBT for six organisms at 6h concentrations of cefazolin plus tobramycin in six spent dianeal fluids.
(*S.epidermidis* isolate #20, *S.aureus* isolate #11, *E.coli* isolate #9, *K.pneumoniae* isolate #2, *E.cloacae* isolate #3, *P.aeruginosa* isolate #30)

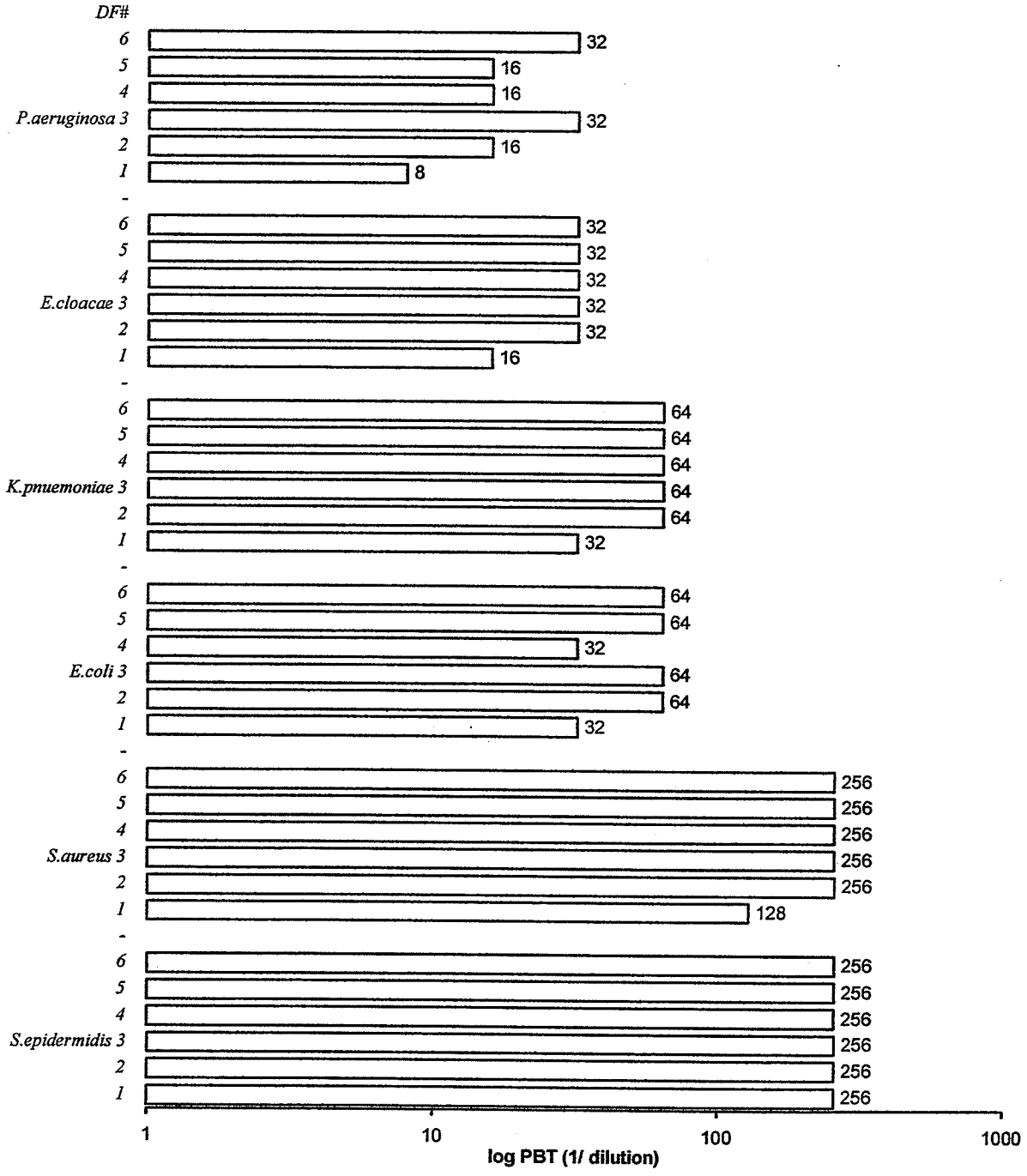
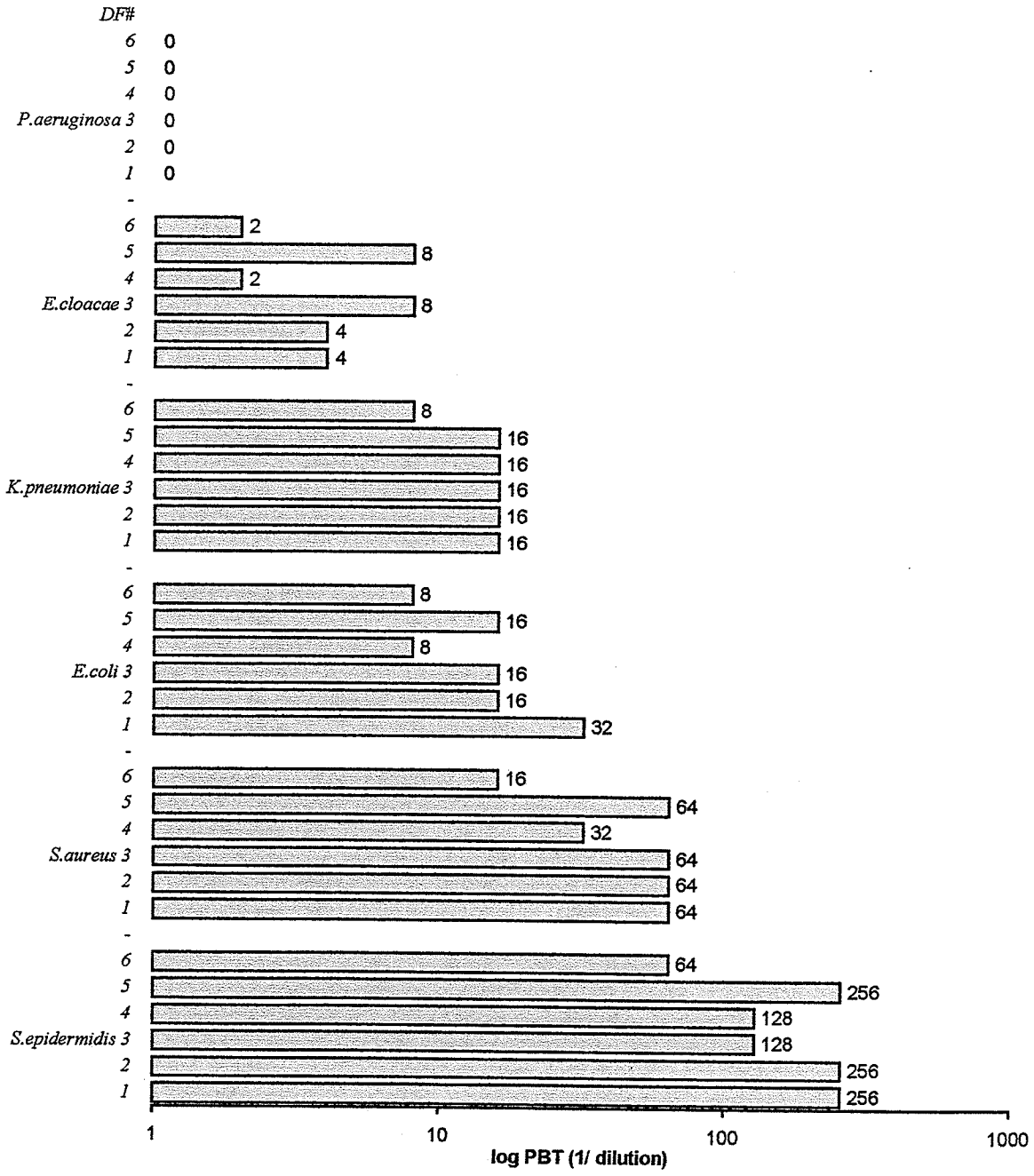


Figure 1b: PBT for six organisms at 24h concentrations of cefazolin plus tobramycin in six spent dianeal fluids.
(*S.epidermidis* isolate #20, *S.aureus* isolate #11, *E.coli* isolate #9, *K.pneumoniae* isolate #2, *E.cloacae* isolate #3, *P.aeruginosa* isolate #30)



PHASE 2: CHARACTERIZATION OF PBT

1. CEFAZOLIN PLUS TOBRAMYCIN PBTs AT 6H AND 24H CONCENTRATIONS

Figures 2a & b show PBTs for all 36 isolates against cefazolin plus tobramycin at 6h and 24 h using one dianeal fluid. PBTs at 6h concentrations were higher than 24h PBTs by one to four fold dilutions. The median PBT for *P.aeruginosa* isolates at 6-h concentration was 1/48 (1/32-1/64) and at 24h concentration all isolates exhibited no inhibition. *E.cloacae* isolates demonstrated activity with cefazolin plus tobramycin. The median 6h PBT for *E.cloacae* isolates was 1/32 (1/20 - 1/32) and 1/3 (½ - 1/7) at 24-h concentrations. PBTs for all *K.pneumoniae* isolates were higher than PBTs for *E.cloacae* isolates. The median PBT for *K.pneumoniae* at 6-h was 1/48 (1/32 - 1/64) and 1/16 (1/10 - 1/16) at 24-h concentrations. A greater amount of PBT variability was seen between *K.pneumoniae* isolates for the cefazolin plus tobramycin regimen than with cefazolin alone. *E.coli* also exhibited similar median PBTs of 1/48 (1/20 - 1/64) at 6-h concentrations and 1/12 (1/8- 1/16) at 24-h concentrations. *S.aureus* isolates exhibited the greatest activity with median PBTs of 1/128 (1/80 - 1/128) at 6-h concentrations and 1/64 (1/64 - 1/112) at 24-h concentrations. *S.epidermidis* also exhibited similar median PBTs of 1/96 (1/64- 1/224) at 6-h concentrations and 1/48 (1/32 - 1/64) at 24-h concentrations.

Figure 2a: Cefazolin plus tobramycin PBTs at 6h against 36 CAPD peritonitis isolates.

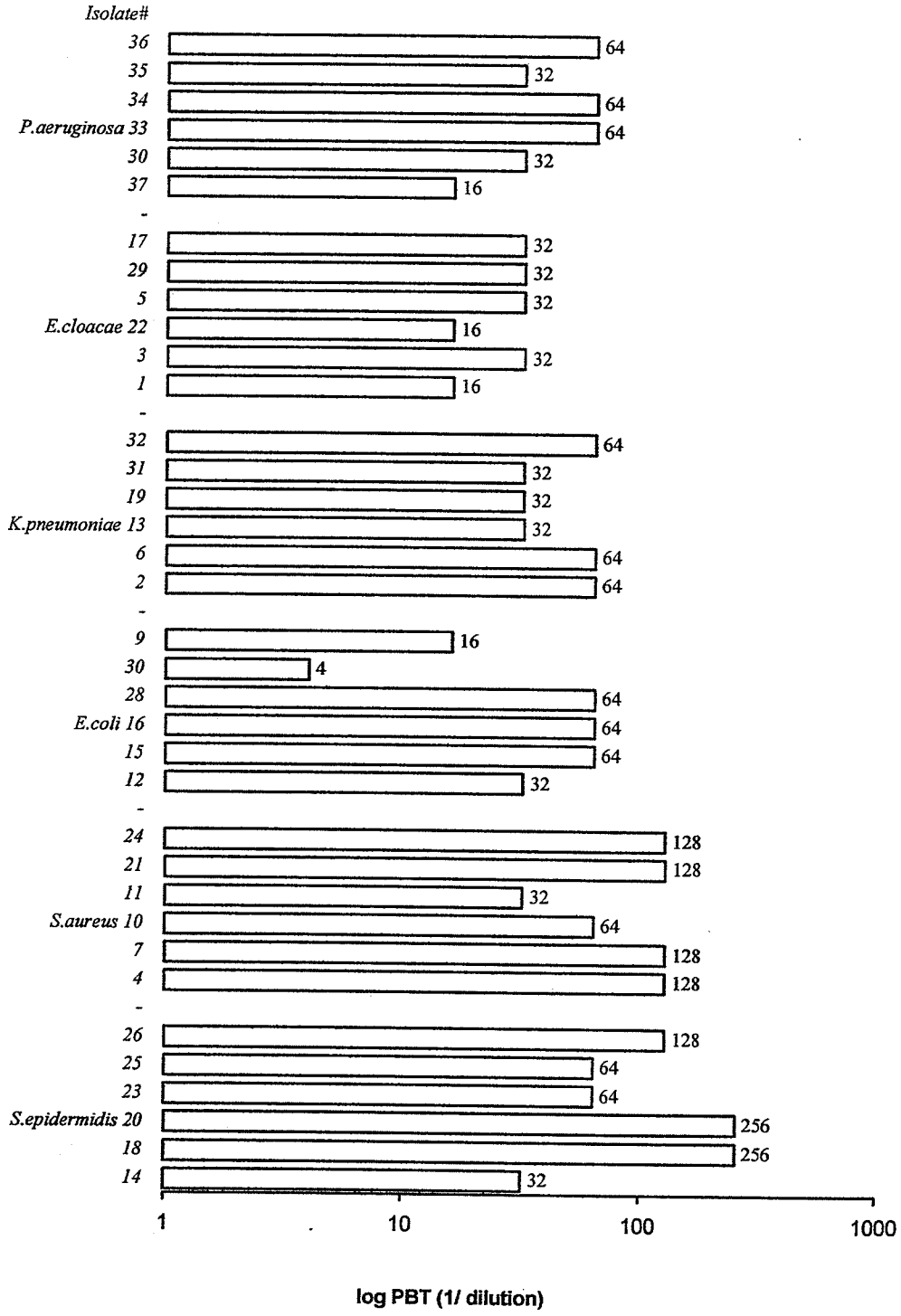
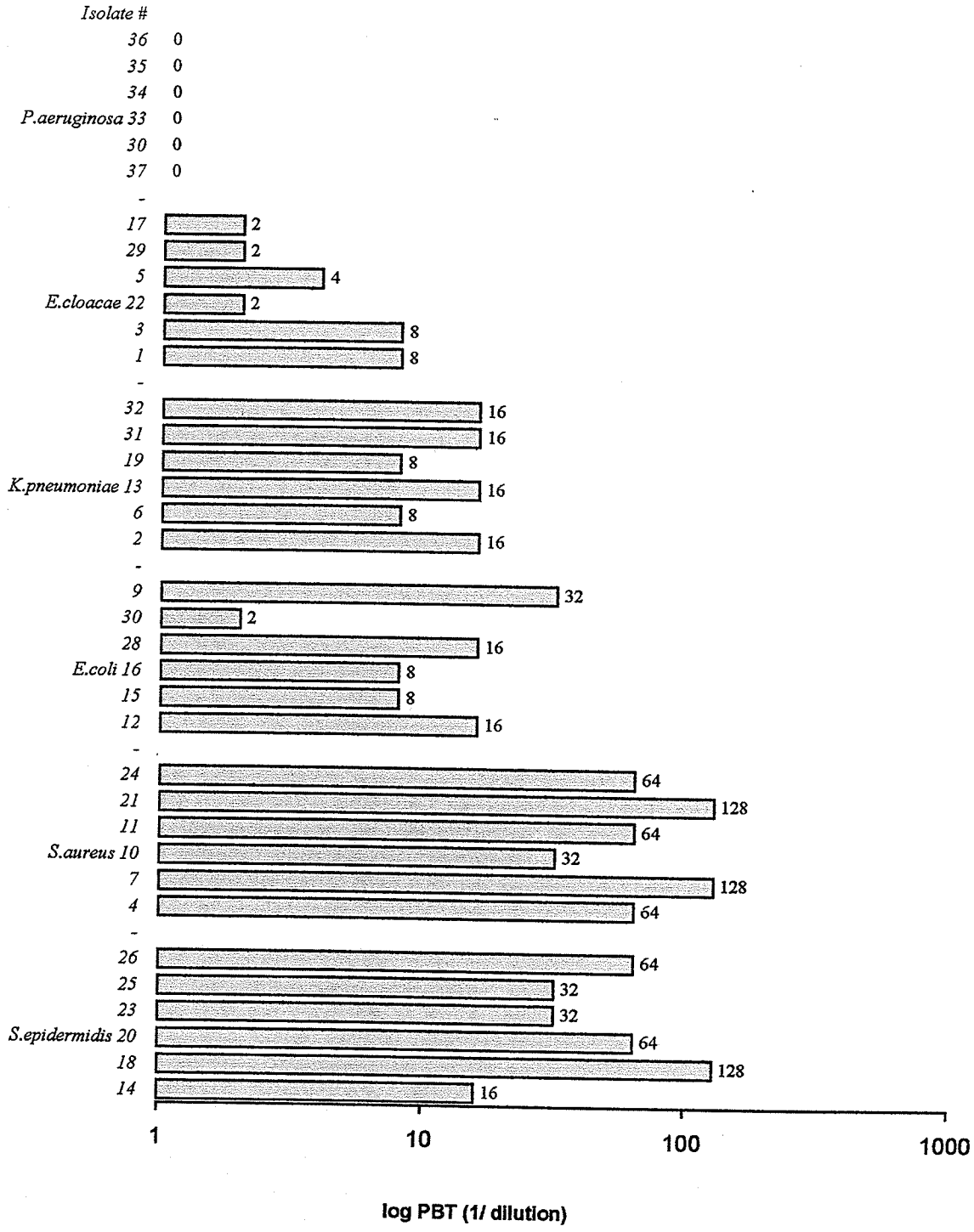


Figure 2b: Cefazolin plus tobramycin PBTs at 24h h against 36 CAPD peritonitis isolates.



2. CEFAZOLIN PBTs AT 6H AND 24H CONCENTRATION

Figures 3a &b show PBTs for all 36 isolates against cefazolin alone at 6h and 24 h using one dianeal fluid. PBTs at 6h concentrations were similar to 24h PBTs for most isolates. *P.aeruginosa* and *E. cloacae* exhibited no inhibition at both 6h and 24h concentrations of cefazolin. *S.aureus* isolates exhibited the greatest activity with a median 6h PBT of 1/128 (1/128) and median 24h PBT of 1/64 (1/40-1/112). *S.epidermidis* at 6h and 24h had median PBTs of 1/48 with an interquantile range of (1/32-1/208) and (1/32– 1/64) respectively. *K.pneumoniae* exhibited the least variability between isolates, at 6h and 24 concentrations median PBTs were 1/32 (1/32) and 1/16 (1/16) respectively. PBTs for five of the 6 *K.pneumoniae* isolates were 1/32 at 6-h concentrations and 1/16 at 24-h concentrations. E.coli exhibited similar PBTs with median PBTs of 1/32 (1/32) at 6h and 1/16 (1/10 – 1/16) at 24h concentrations.

Figure 3a: Cefazolin PBTs at 6h against 36 CAPD peritonitis isolates

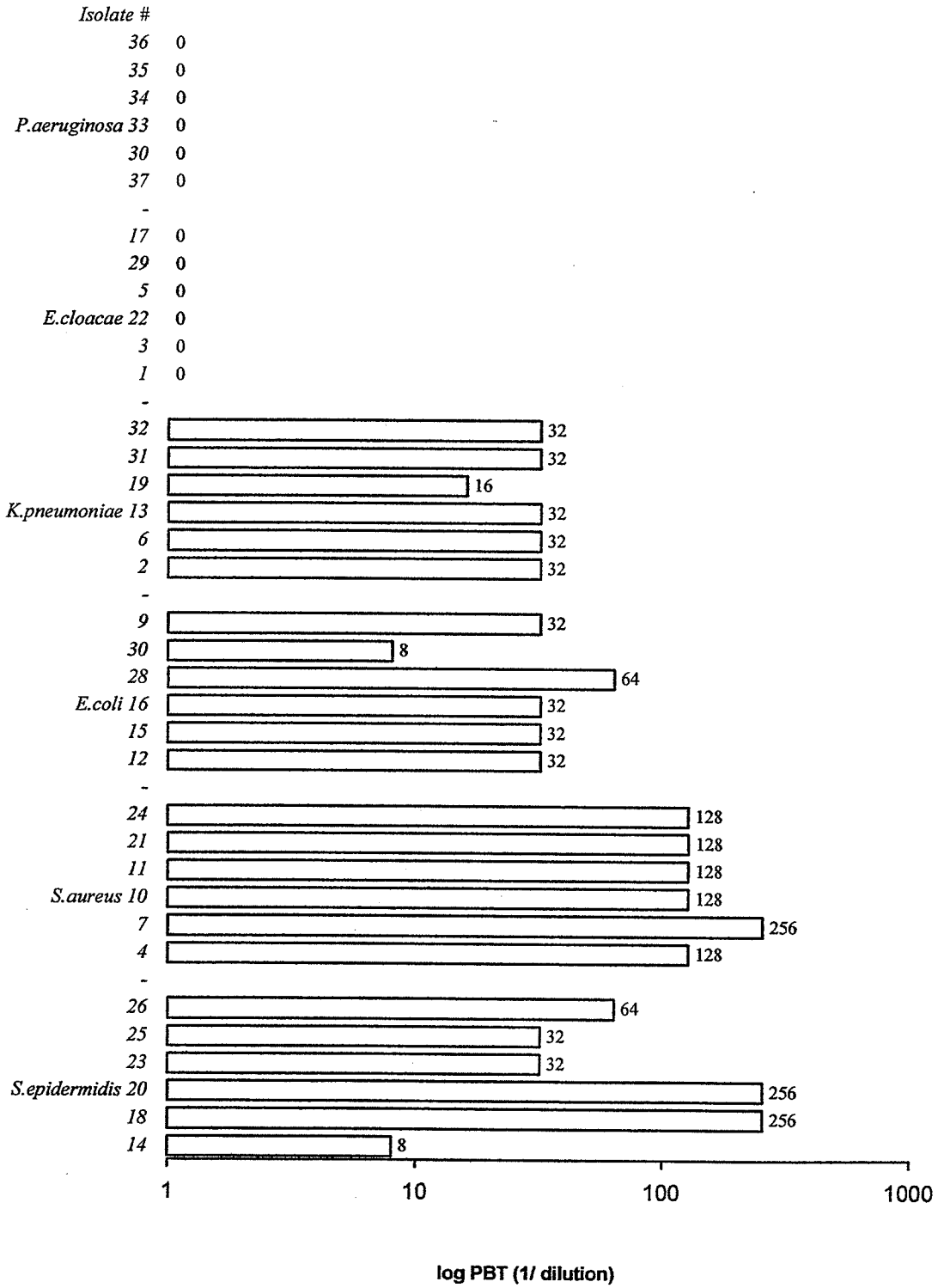
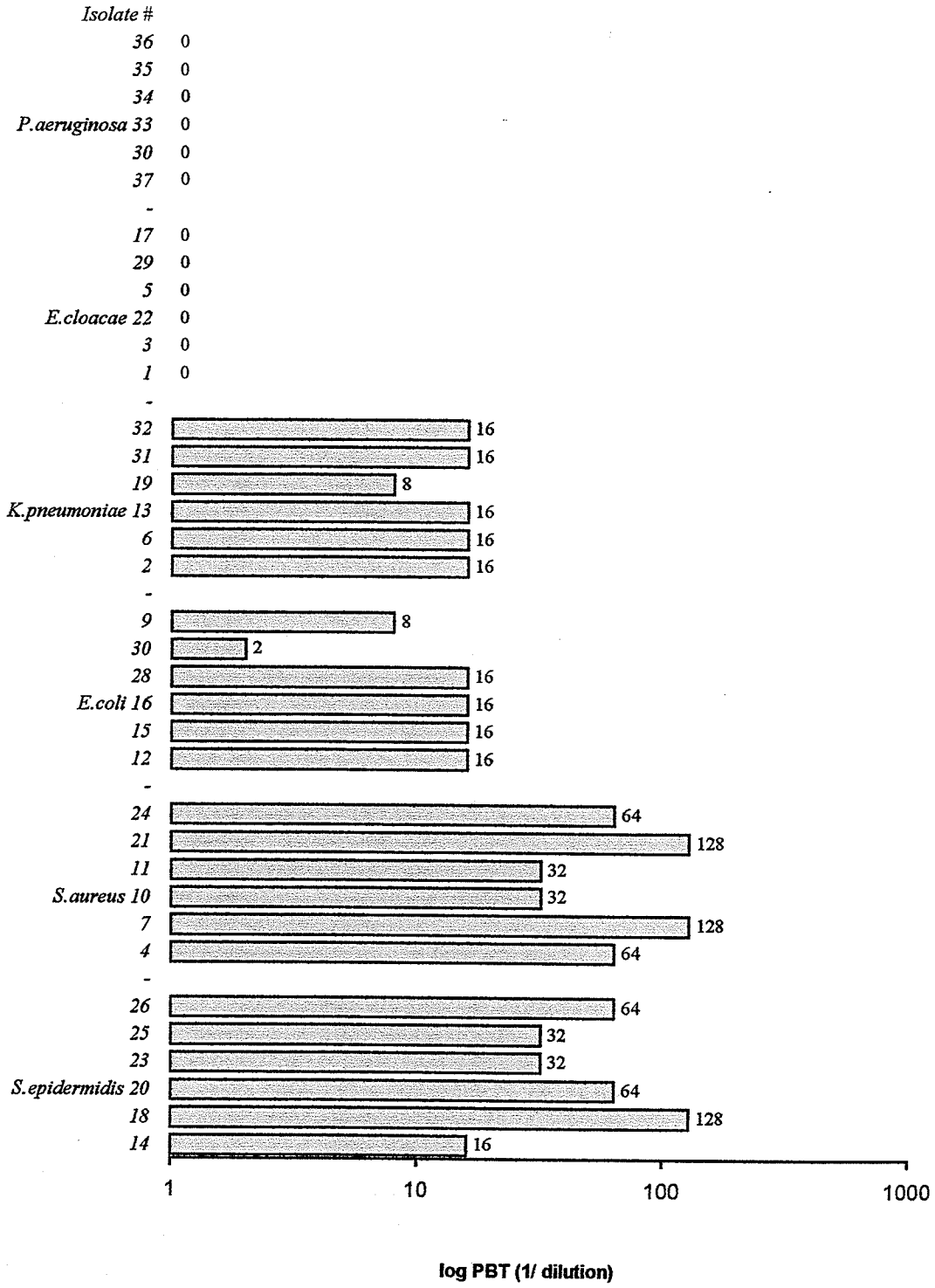


Figure 3b: Cefazolin PBTs at 24h against 36 CAPD peritonitis isolates



3. PBTs AT 6H VERSUS 24H CONCENTRATION

Figure 4a demonstrates the differences in activity between 6h and 24h concentrations with the cefazolin plus tobramycin regimen. PBTs for most isolates at 6h concentrations increased 4-fold to 64-fold. The Mann-Whitney test detected a significant difference in overall activity between 6h and 24 h concentrations for cefazolin plus tobramycin ($p>0.0001$) The most remarkable change in PBTs between 6h and 24h concentrations was seen for *P.aeruginosa* and *E. cloacae* isolates. All six *P.aeruginosa* isolates had 16-fold to 64- fold increases in PBTs at 6h concentrations compared to 24h. Five *E.cloacae* isolates had 4-fold to 16-fold increases in PBTs at 6 h concentrations. Three *E.coli* isolates and four *K.pneumoniae* isolates exhibited 4-fold to 8-fold increases in PBTs at 6h concentrations. PBTs for all *S. aureus* isolates and four *S.epidermidis* isolates remained unchanged between the two concentrations, apart from two *S.epidermidis* isolates in which PBTs increases four fold at the 6 h concentration.

Figure 4b compares activity between 6-h and 24-h concentrations of the cefazolin regimen. The Mann-Whitney test did not detect significant differences in overall PBTs between the two concentrations. ($p=0.197$) There was a four fold increase in PBTs with 6h concentrations exhibited for three *E.coli* isolates, two *S. aureus* isolates and one *S.epidermidis* isolate.

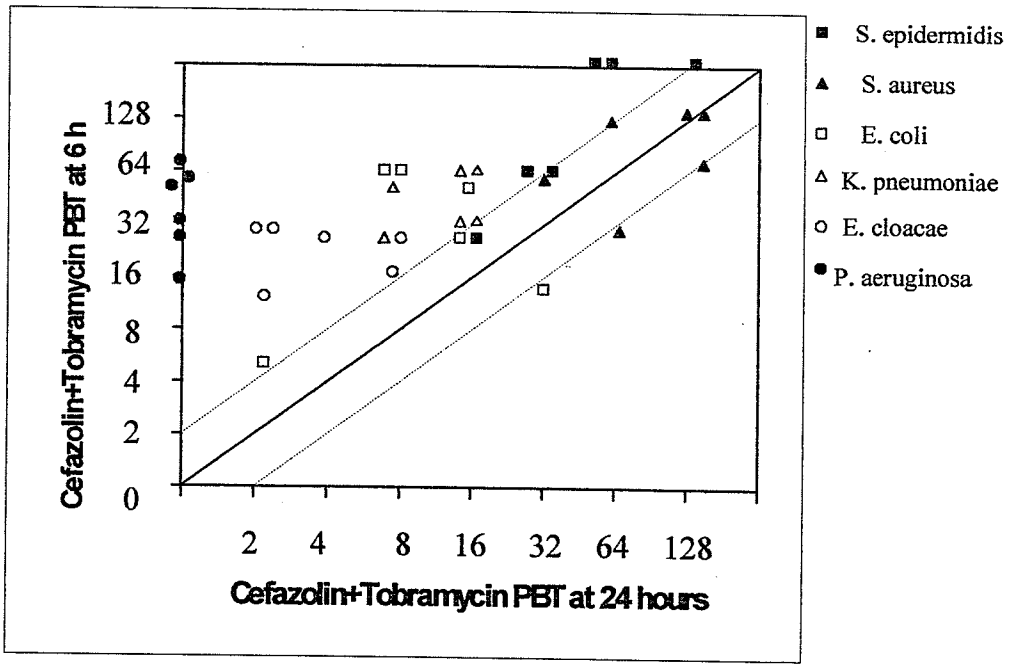


Figure 4a: PBTs for cefazolin plus tobramycin at 6 and 24h concentrations. (p= <0.0001)
(———— line of unity; - - - - - - PBT values within one dilution)

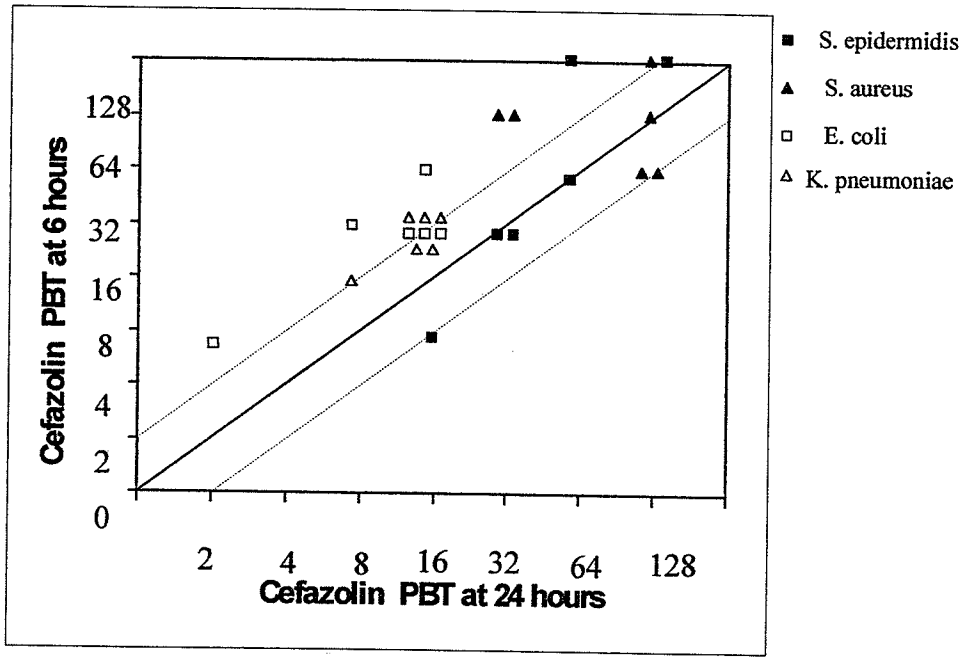


Figure 4b: PBTs for cefazolin at 6 and 24-h concentrations. (p=0.197)
(———— line of unity; - - - - - - PBT values within one dilution)

4. CEFAZOLIN VERSUS CEFAZOLIN PLUS TOBRAMYCIN PBTs AT 6H AND 24H

A comparison of cefazolin to cefazolin plus tobramycin treatment regimens at 6 h is presented in Figure 5. PBTs at 6-h concentration for cefazolin alone were plotted against PBTs for cefazolin plus tobramycin. The Mann-Whitney test detected a significant difference in overall activity between these to regimens. (p=0.012) With a margin of error of +/- one dilution one isolate of *S.epidermidis* demonstrated a 4-fold increase in PBTs with cefazolin plus tobramycin. All six isolates each of *E. cloacae* and *P.aeruginosa* exhibited a 16-64-fold increase in measured PBTs with cefazolin and tobramycin compared to cefazolin alone. Five *S. aureus* isolates and all 6 *K.pneumoniae* and *E.coli* isolates demonstrated no difference in activity between the two treatment regimens at 6-h concentrations. One isolate each of *S.aureus* and *S.epidermidis* demonstrated a four fold increase in PBTs with cefazolin alone.

Measured PBTs at 24 h concentrations for cefazolin alone were plotted against PBTs for cefazolin plus tobramycin as shown in Figure 6. The Mann-Whitney test did not detect a significant difference between the two regimens at 24h. (p=0.634) With a margin of error of +/- one dilution, all six isolates each of *S. epidermidis, S. aureus* and *K. pneumonia* demonstrated no difference in activity between the two treatment regimens at 24-h concentrations. One *E. coli* isolate and two *E. cloacae* isolates demonstrated a four fold increase in measured PBTs with cefazolin and tobramycin.

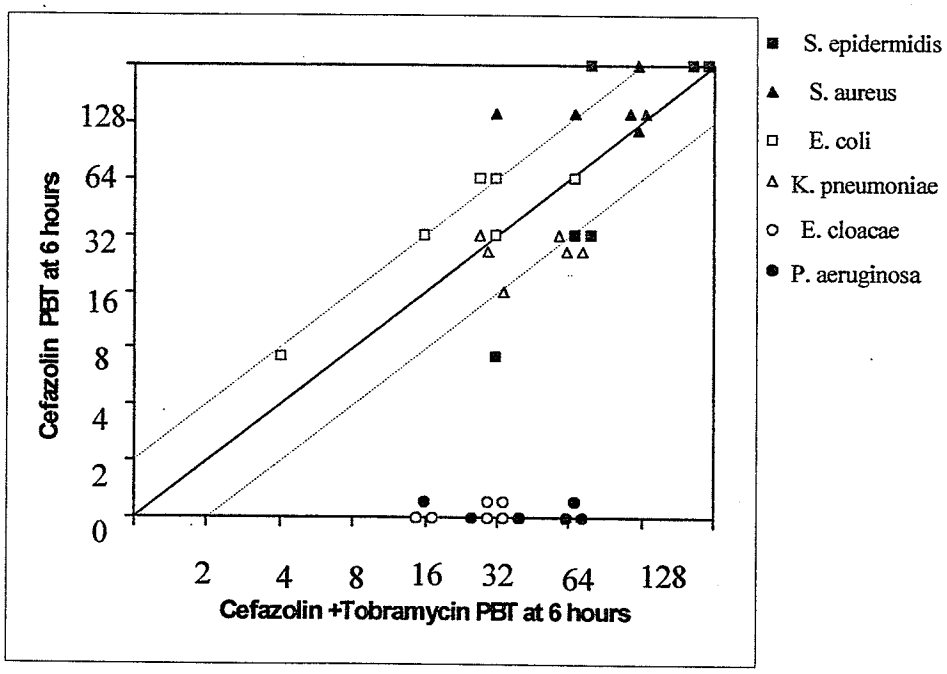


Figure 5: PBTs for cefazolin and cefazolin plus tobramycin at 6-h concentrations. (p=0.012)
(———— line of unity; - - - - - - PBT values within one dilution)

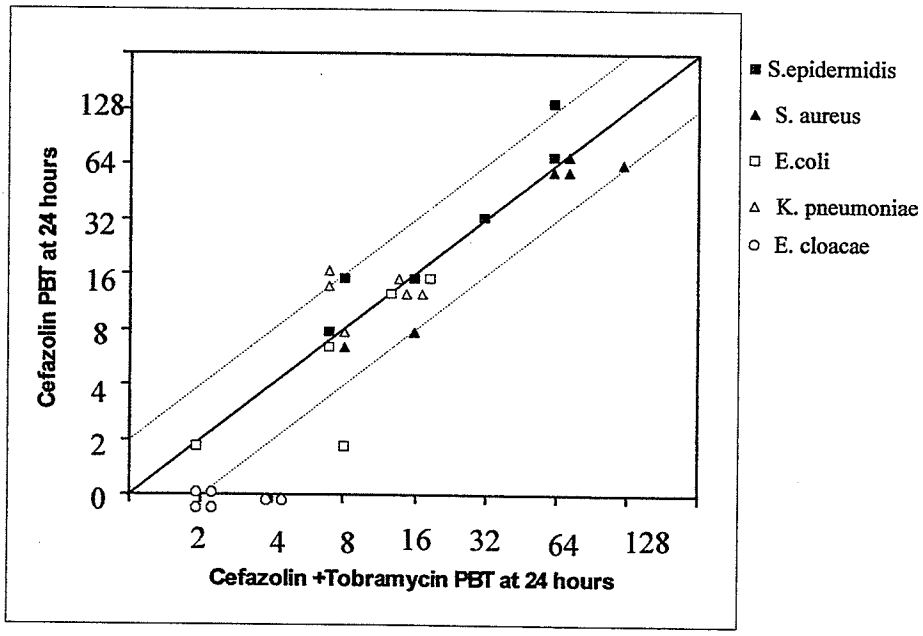


Figure 6: PBTs for cefazolin and cefazolin plus tobramycin at 24-h concentrations. (p=0.634)(———— line of unity; - - - - - - PBT values within one dilution)

5. PBTs COMPARED TO TRADITIONAL MICROBIOLOGICAL TEST (MBC)

In order to asses the correlation of PBTs to the current standard of MBC measurements in broth, 6h PBTs for cefazolin alone were compared to calculated titre, by dividing the predicted concentration of cefazolin at 6h by the MBC for cefazolin for each as shown in Figure 7a. All six *E.cloacae* and *P.aeruginosa* isolates demonstrated no inhibition of growth with predicted 6-h cefazolin concentrations in dianeal fluid and therefore are not shown. With a margin of error of +/- one dilution, measured PBT results for all six isolates each of *E. coli* and *K. pneumoniae* corresponded with calculated PBT results at 6 h. At 6 h, one *S.epidermidis* isolate exhibited an eight fold increase in measured activity in dianeal fluid and four *S.aureus* isolates exhibited 16, eight and four fold increases in activity in dianeal fluid.

The correlation of PBTs to calculated titres based on current standard of MBC measurement in broth at 24h concentrations for cefazolin alone are shown in Figure 7b. As above, calculated PBTs were obtained by dividing the predicted concentration of cefazolin at 24h by the MBC. Correlation results were similar to 6h PBT data. All six *E.cloacae* and *P.aeruginosa* isolates demonstrated no inhibition of growth with predicted 24-h cefazolin concentrations in dianeal fluid. With a margin of error of +/- one dilution, measured PBT results for all six isolates each of *E. coli* and *K. pneumoniae* corresponded with calculated PBT results at 24 h. At 24 h, two *S.epidermidis* isolates exhibited a four

fold and 16-fold increase in measured activity in dianeal fluid and three *S.aureus* isolates exhibited 16, eight and four fold increases in activity in dianeal fluid. None of the isolates demonstrated a decrease in activity in dianeal fluid.

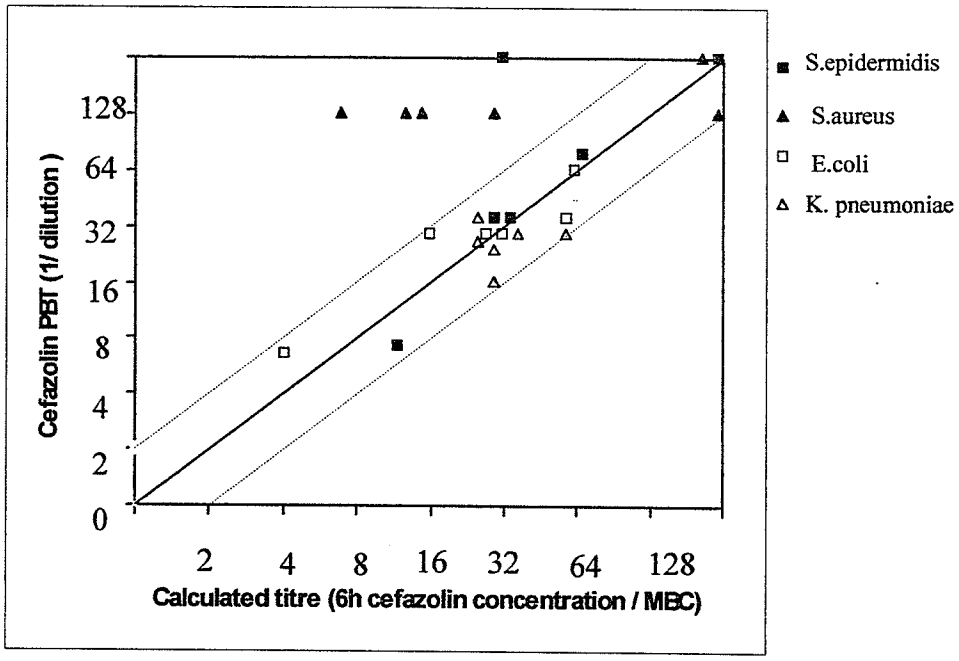


Figure 7a: PBTs versus calculated titres based on antibiotic concentration at 6h divided by MBCs.
(———— line of unity; - - - - - - PBT values within one dilution)

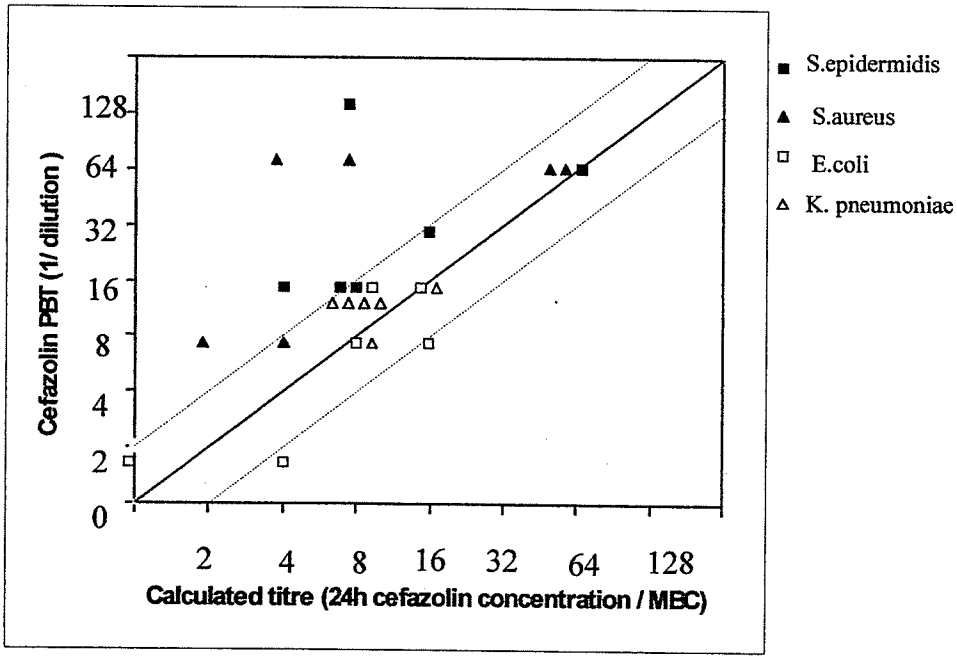


Figure 7b: PBTs versus calculated titres based on antibiotic concentration at 24h divided by MBCs. (———— line of unity; - - - - - - PBT values within one dilution)

PHASE 3: PILOT PBT STUDY

Table 8 summarizes PBT data and outcome data for 14 patients treated for CAPD peritonitis. Of the 14 patients 50% were male and 50% were female. The average age (+/- SD) at the time of peritonitis was 64+/-12 years. The causative organisms consisted of two *Klebsiella* species, three *Coagulase negative staphylococci,* two *E.coli*, two *S.aureus,* two *S.epidermidis* isolates and one of each of *S.capitis, Aeromonas hydrophila* and *Stenotrophomonas maltophilia.* Of the 14 patients, ten patients were initially started on empiric cefazolin 1.5g once daily and tobramycin 60mg once daily added to a 2 L bag. Of the ten patients started on empiric therapy, three patients failed treatment. Failure was defined as either a reoccurring infection within 14 days, catheter removal and transfer to hemodialysis, death or change in therapy excluding changes due to allergy.

Two patients were started on vancomycin 2g once weekly and tobramycin 60mg once daily added to a 2L bag and both patients were successfully treated. One patient was started on cefazolin 1.5g once daily and tobramycin 60mg once daily added to a 2L bag with the addition of ceftazidime 125mg to each 2L bag and was successfully treated. One patient was started on cefazolin 1.5g once daily plus ceftazidime 125mg to each 2L bag and failed treatment. At 6h concentrations, the median PBT for patients with resolved peritonitis was 1:96 (1/64– 1/224), whereas the median PBT for patients who failed treatment was 1:32 (1/24.5-1/40). At 24h concentrations, the median PBT for patient with resolved peritonitis was 1:24(1/16-1/56) whereas the median PBT for patient who failed treatment was 1/4 (1/3-1/7). Significantly less antibacterial activity was observed in patients who failed treatment for both 6h and 24h. (p=0.036)

Table 8: Pilot PBT study in 14 patients treated for CAPD peritonitis.

Patient	Gender	Age	Organism	[a] Intraperitoneal Antibiotic regimen (MIC/MBC in ug/ml)	PBT (6h)	PBT (24h)	Outcome
1	F	50	*K. pneumoniae*	Cefazolin (8/8) + Tobramycin (16/16)	1:64	1:16	Failed
2	M	65	*S.maltophilia*	Cefazolin (>256) + Ceftazidime (64/128)	1:2	0	Failed
3	F	52	*E.coli*	Cefazolin (16/32)+ Tobramycin (8/8)	1:32	1:4	Failed
4	F	52	*E.coli*	Cefazolin (8/32)+ Tobramycin (8/8)	1:32	1:4	Failed
5	M	70	*Coag. neg. staph.*	Cefazolin (1/1) + Tobramycin (32/32)	1:64	1:32	Resolved
6	F	71	*Aeromonas hydrophila*	Cefazolin (>32)+ Tobramycin (16/16)	1:16	1:2	Resolved
7	F	71	*S. capitis*	Cefazolin (64/128) + Tobramycin (0.25/0.5)	1:64	1:64	Resolved
8	F	79	*Coag. neg. staph*	Cefazolin (2/2)+ Tobramycin (1/2)	>1:256	1:64	Resolved
9	F	60	*S.aureus*	Cefazolin (2/2)+ Tobramycin (2/2)	1:128	1:64	Resolved
10	F	83	*S.epidermidis*	Cefazolin (64/64) + Tobramycin (1/2)	1:128	1:32	Resolved
11	M	45	*S.epidermidis*	Cefazolin (64/64)+ Tobramycin (1/1)	1:64	1:16	Resolved
12	F	72	*K. pneumoniae*	Cefazolin (8/8) + Tobramycin (1/2) + Ceftazidime (16/32)	1:64	1:16	Resolved
13	M	72	*Coag. neg. staph*	Vancomycin (4/8)+ Tobramycin (1/2)	>1:256	1:8	Resolved
14	M	71	*S.aureus*	Vancomycin (4/8)+ Tobramycin (8/32)	>1:256	1:16	Resolved

[a] Doses: Intraperitoneal Cefazolin 15g/ 2L once daily
Intraperitoneal Tobramycin 60mg/2L once daily
Intraperitoneal Ceftazidime 125mg/L in every bag
Intraperitoneal Vancomycin 2g/ 2L every 7 days

DISCUSSION

The PBT was developed to assess antibiotic pharmacodynamics in spent dianeal fluid collected from patients with CAPD peritonitis. Unlike MIC and MBCs, which reflect antibiotic activity in broth, the PBT takes into account dianeal fluid composition and its effects on bacterial growth and antibiotic activity. The PBT also measures the overall antibacterial effect of antibiotic combinations.

Phase 1: PBT development:

The methodology of the PBT was modified into an efficient and accurate (+/- one dilution) procedure for assessing antibacterial activity in dianeal fluid. The methodology demonstrated reproducible results when preformed by different individuals. The test is relatively quick, requiring a small amount of microbiology lab time. (Approximately 10-minutes/ sample) In this study dianeal fluid was spiked with antibiotics so as to eliminate variability in PBTs due to inter-patient variability in dialysate antibiotic concentrations.

Processed dianeal fluid stored at room temperature or incubated at 35°C with or without the addition of antibiotics formed an oily precipitate following 24h. The precipitate was plated in all cases to eliminate contamination as a possible cause of cloudiness. The formation of precipitate did not inhibit antibiotic activity to any significant extent since antibacterial activity was exhibited in serial dilutions of the initial

cloudy tube with all sensitive organisms. Throughout the study, the first tube, which contained 100%, spent dianeal fluid, spiked with antibiotic and inoculated, formed a precipitate following 24h incubation. All subsequent serially diluted tubes (i.e.: tubes two to 8) were clear unless growth occurred due to the serial reduction antibacterial activity. Therefore since tubes two to eight did not contain any precipitates, PBT endpoints could be determined from these tubes by visual assessment. If however, tubes two to eight were cloudy due to a lack of antibacterial activity, the first tube would be plated to determine if viable organisms were present. This method of PBT endpoint determination was suitable since the majority of PBTs were well above a 1/2 dilution for sensitive isolates. The formation of precipitate was not due to an interaction with the spiked antibiotic as it occurred with or without antibiotic. Dianeal fluid diluted 1:1 with broth did not form a precipitate, since tubes two to eight had no precipitate formation. Wilcox et al. 1990 also observed the formation of precipitate in spent dianeal fluid. Dianeal fluid exposed to air, at room temperature or with incubation at 37°C resulted in a mean increase in pH of 1.23 U and formation of protein precipitate. The same study also noted a larger precipitate yield in dialysate incubated at 37°C than in dialysate incubated at room temperature. In the development of the PBT the initial concern was the possibility of precipitation due to the interaction of antibiotic with the dianeal fluid. In the PBT study an oily precipitate was only present in 100% dianeal fluid and did not affect antibiotic activity given that bacterial inhibition of sensitive organisms was observed in subsequent tubes.

Biochemical analysis was preformed on all dianeal fluids. Electrolyte variability between different dianeal fluids was seen with the least amount of difference in measured calcium concentrations. Fresh dianeal fluid contains added amounts of electrolytes for normalization of serum electrolyte concentrations. Most commercially available dianeal fluids contain added amounts of calcium, magnesium, sodium chloride and sodium lactate. Electrolytes are added to reproduce physiological concentrations and dianeal fluid variability between patients is dependent on the ability of electrolytes to cross the peritoneal membrane and on the serum electrolyte concentrations of the patient. The high degree of variability in glucose concentrations (Table 3) can be attributed to the type of dianeal fluid and patient blood glucose levels. Dianeal fluids contain varying amounts of glucose as the osmotic agent. However, depending on the patient, glucose can be rapidly absorbed leading to hyperglycemia. The wide range in glucose between spent dianeal fluids (i.e.: 1.5 to 55.5 mmol/L) is reflective on variability in the types of dianeal fluid used. A high degree of variability in protein concentrations was also exhibited between the spent dianeal fluids. Depending on the state of the peritoneal membrane there is a gradual loss of protein into the dialysate during the dwell period. Amino acid absorption through the use of amino acid containing dianeal fluids has been linked to vasodilatation within the peritoneal membrane thus increasing surface area and protein loss into the dialysate. (Steinhauer, Lubrich-Birkner et al. 1992) The average pH of dianeal fluid was 7.4 and there was little variability between spent dianeal fluids. During the dwell, the composition and pH of the dialysis fluid becomes more physiologic therefore we would expect the pH to approach 7.4. Dianeal fluid pH was measured prior to processing and incubation, therefore the pH values in Table 3 represent intraperitoneal

pH levels and do not reflect increases in pH which has been observed in dianeal fluid from of the studies in which precipitation occurred when spent dianeal fluid was exposed to air and incubated at 37°C. (Wilcox, Smith et al. 1990)

Dianeal fluid storage at –70°C for greater than six months resulted in significant changes in dianeal fluid composition. A decrease in calcium levels and increase in magnesium and creatinine levels was observed following a 12 month storage period. For PBT testing, dianeal fluid can be stored at –70°C for a maximum six month duration.

Growth studies in dianeal fluid were preformed prior to developing the PBT. Growth in M-H broth was compared to growth in 50% of spent dianeal fluid. All isolates (*S.epidermidis, S.aureus, E.coli, E.cloacae, K.pneumoniae,* and *P.aeruginosa*) grew well in spent dianeal fluid, apart from alpha-hemolytic streptococci isolates, which did not grow in spent dianeal fluid. Growth studies were preformed with two different dianeal fluids (DF2, DF3) in Table 3. The growth study results coincide with results from a study preformed by Wilcox et al. 1990 in which coagulase negative staphylococci exhibited growth in spent dianeal fluid when exposed to air and incubated at 37°C. Wilcox et al. reported less extensive growth of coagulase negative staphylococci in spent dianeal fluid when exposed to air versus growth in a CO_2 environment. Coagulase negative staphylococci growth is said to be affected by increases in pH and calcium levels in dialysate. (Wilcox, Smith et al. 1990; Morton, Evans et al. 1994) The calcium concentrations of dianeal fluids (DF2 and DF3) used in the growth studies were 1.38 and 1.19 mmol/L with a pH of 8.0 and 7.0 respectively. Coagulase negative isolates demonstrated grow in spent dianeal fluid, however the effects of calcium concentration

and pH on organism growth were not detected. Macdonald et al. 1986 examined the effects of dianeal fluid composition and pH on *S.epidermidis* growth and observed that proteins present in dianeal fluid following a minimum 2 h dwell also promoted the growth of *S. epidermidis.* Likewise, the same study demonstrated a reduction in S. epidermidis growth with acidification of dianeal fluid to less then pH 6.35. There were no differences in growth for one of the two *S. epidermidis* isolates observed between the two samples of dianeal fluid despite protein levels of DF2 being approximately double the concentration found in DF3 (i.e.: 1.280 versus 0.512 mg/L respectively) The second *S.epidermidis* isolate did however exhibit greater growth in DF2, it is also important to note that DF2 contained more protein but also had a higher pH. The difference in *S.epidermidis* growth in the two samples of dianeal fluid isolates may be isolate dependent. A study by Sheth et al. 1986 also reported extensive coagulase negative staphylococci and *S. aureus* growth up to 48h, however both organisms did not survive in spent dianeal fluid following an incubation period of 96h. The same study also reported extensive growth of *E.coli* and *P.aeruginosa* in spent dianeal fluid over 24h, which also coincided with growth study results.

Dianeal fluid was not used as a diluent in this study. The PBT was developed as a method of measurement of both the intrinsic antibacterial activity of the dianeal fluid itself in addition to the activity of the antibiotic regimen within the dianeal fluid. As discussed, serial dilution with both allowed for a measurement of both components. The combined antibacterial effect present at specific dilutions provided a method of measuring overall antibacterial effect. The intrinsic effect of dianeal fluid was observed

when PBT were compared between different samples of spent dianeal fluid. The least amount of difference between dianeal fluids was detected at 6h antibiotic concentrations. Failure to measure difference in dianeal fluid activity at 6h may be due to the high levels of antibiotic at this time point and the corresponding optimum antibacterial activity. Therefore antibacterial activity measured at the 6h time point is a result of high antibiotic concentrations and antibiotic activity in dianeal fluid. Changes in dianeal fluid composition did not have a measurable effect on antibacterial activity at this time point. Reduction of antibiotic concentrations at 24h resulted in an overall reduction in antibacterial activity due to antibiotic alone. For *P.aeruginosa*, effects of dianeal fluid on PBTs were observed at 6h due to the relatively poor activity of cefazolin plus tobramycin. However, more organisms exhibited changes in antibacterial activity between samples of dianeal fluid at 24h antibiotic concentration. At this time point antibiotic concentrations were lower and this allowed for measurement of the effects of dianeal fluid on antibacterial activity. Dianeal fluid variability accounted for a two-fold difference in PBTs, and each organism was affected by variations dianeal fluid composition differently. Studies examining the effect of dianeal fluid have attributed factors such as calcium concentration, pH, electrolytes, protein content and osomolarity on growth and antibiotic activity of various organisms. (Duwe, Vas et al. 1981; McDonald, Watts et al. 1986; Wilcox, Smith et al. 1990; Thomas, Schenk et al. 1997) In this study, further examination of dianeal fluid composition and PBT activity failed to detect any link of antibacterial activity to dianeal fluid biochemistry or pH. This would be expected since the effect of dianeal fluid in most cases is isolate specific.

End point determination by visual inspection was chosen instead of performing bactericidal counts given that in approximately 90% of cases inhibition of growth (i.e.: first clear tube) and bactericidal counts were equal or differed by +/- one dilution. Therefore, taking into account the accuracy of the PBT test and in order to simplify PBT testing, endpoints were determined by visual inspection alone. Studies examining the effect on dianeal fluid on antibacterial activity against *P.aeruginosa* isolates have documented the occurrence of tolerance (i.e.: MIC: MBC >32) against ceftazidime and piperacillin antibiotics. (Zelenitsky, Franczuk et al. 2002) In this study PBTs for *P.aeruginosa* isolates were tested against cefazolin plus tobramycin and cefazolin alone regimens. For both regimens, the effect of tolerance was evaluated by comparing PBT assessment by visual endpoint determination (inhibitory endpoints) versus bactericidal counts (bactericidal endpoint) for *P.aeruginosa* isolates. For all isolates inhibitory and bactericidal endpoints were similar and therefore tolerance was not observed for these regimens. For the regimens tested, visual endpoint determination would not have missed the effect of tolerance. Perhaps assessing regimens that contain antibiotics such as ceftazidime and piperacillin, where tolerance has been documented, PBT endpoint determination by bactericidal counts may be required.

Phase 2: Characterization of the PBT

The PBT results for various organisms were reflective of treatment failure rates obtained from the literature. Cefazolin plus tobramycin PBTs were highest for *S.aureus* isolates (median of 1/128 at 6h and 1/64 at 24h). *S.aureus* peritonitis is known to respond

well to antibiotics and has a relatively low failure rate of 20%. (Goldberg, Clemenger et al. 2001) *P.aeruginosa* isolates demonstrated the lowest PBTs for the cefazolin plus tobramycin regimen. (median of 1/48 at 6h and 0 at 24h) This also corresponds with difficult to treat Pseudomonal infections, with failure rates being as high as 50%. (Piraino, Bernardini et al. 1987) Each isolate exhibited a characteristic range of PBTs.

PBTs measured greater activity at 6h concentrations than 24 h concentrations. For once daily administration of cefazolin plus tobramycin, antibiotic are added to the first bag and all subsequent exchanges of dianeal fluid throughout a 24h period would not contain any additional antibiotic. For 6h dwell periods, 24h PBTs reflect the end of dwell antibiotic levels achieved following a fourth bag exchange within at 24 h period and represent the least amount of activity exhibited with one daily dosing. The first bag to which antibiotic are added (i.e.: first 6h dwell) would therefore contain higher antibiotic concentrations thereby effectively producing the greatest amount of kill within the first 6 h. The measurement of antibacterial activity at this time point reflects the effect of antibiotic activity more so then the intrinsic effects of dianeal fluid. A 6h PBT provides information on antibiotic sensitivity, the effect of antibiotic combinations, antibiotic absorption kinetics (: i.e. antibiotic concentrations in dianeal fluid) and the effect of dianeal fluid on antibacterial activity. Starting antibiotic concentrations for PBTs at 24h are much lower and subsequent serial dilutions reflect smaller changes in antibiotic concentration. PBTs at 24h consist of smaller antibiotic dilutions thereby increasing the capability of detecting smaller changes in antibacterial activity. At the 24h time point, intraperitoneal kinetic consideration such as the permeability of the peritoneal membrane,

extent of initial absorption following the first dose and subsequent re-absorption back into the peritoneal cavity with repeat exchanges can produce a larger variability in 24h antibiotic concentrations. PBTs obtained at 24h time points provide information on the extent of antibacterial activity throughout the course of treatment. PBT at 24h can measure smaller changes in antibacterial activity due to antibiotic activity and detect differences in antibacterial effects between dianeal fluids.

Scattergrams were extensively used in PBT analysis. Scattergrams were used to asses differences in PBTs between time points and treatment combinations and were used in comparing PBTs to MBCs. PBTs for cefazolin plus tobramycin at 6h were higher than PBTs at 24 h. However for *S.epidermidis* and *S.aureus* optimum antibacterial activity was achieved at 24h concentrations and was not improved at 6h. Cefazolin is a time dependent antibiotic therefore organisms such as *S. epidermidis* and *S.aureus*, which are sensitive to cefazolin, would not exhibit greater activity at 6h concentrations. This was exhibited with cefazolin alone at 6 and 24h. However for Gram-negative organisms such as *E. coli*, *K.pneumoniae*, *E.cloacae* and *P.aeruginosa* there was a greater increase in activity at 6h concentrations of cefazolin plus tobramycin. *P.aeruginosa* isolates demonstrated relatively good activity at 6h for cefazolin plus tobramycin, however there was no inhibition of growth at 24h concentrations. This is not attributed to the increase in cefazolin concentration but more so the effect of increasing concentrations of tobramycin. Tobramycin is a concentration dependent antibiotic, therefore an increase in activity would occur with increasing concentrations. It is difficult is select which time

point would be most beneficial in assessing antibacterial activity as both time points provide important information.

Scatter grams were also used to compare PBTs between regimens of cefazolin plus tobramycin and cefazolin alone and 6h and 24h. The use of PBTs for assessing combination therapy would be very applicable in the evaluation of new treatment regimens since CAPD peritonitis is often treated with two antibiotics. . PBTs for cefazolin plus tobramycin regimens were higher then PBTs for cefazolin alone at 6h for *E. cloacae* and *P.aeruginosa.* (Figure 5) For *P.aeruginosa* and *E.cloacae* there was an added benefit seen with the addition of tobramycin particularly at 6h concentrations. Both organisms exhibited no inhibition with cefazolin alone since all *E.cloacae* and *P.aeruginosa* isolates exhibited poor activity with cefazolin. Therefore the increases in antibacterial activity observed with the addition of tobramycin were primarily due to sensitivity to tobramycin. *E.cloacae* PBTs at 24h were higher with the addition of tobramycin for only two isolates and P.aeruginosa did not exhibit any inhibition of growth at 24h concentrations of cefazolin plus tobramycin. The MICs for *P.aeruginosa* and *E cloacae* were between two to 4 ug/mL, therefore the addition of tobramycin 3.2ug/mL (24h concentration) would not significantly increase antibacterial activity. Gram-negatives such as *E. coli* and *K.pneumoniae* produced relatively high PBTs with cefazolin alone therefore the addition of tobramycin at 6 h concentrations, did not significantly increase PBTs. Only one *E.coli* isolate exhibited an increase in PBTs with the addition of tobramycin at 24h concentrations. This isolate had MICs for cefazolin and tobramycin of 4 ug/mL and exhibited relatively poor activity with cefazolin alone.

The addition of tobramycin despite being close to MIC levels contributed to an increase in overall antibacterial activity.

The addition of tobramycin, particularly for the Gram-positive organisms, did not affect PBTs at 6h and 24h for organisms optimally treated with high concentrations of cefazolin. Tobramycin therapy of <two days in the treatment of *S. epidermidis* peritonitis has been associated with treatment failure. (Ariano, Franczuk et al. 2002) A small increase in PBTs with the addition of tobramycin was observed with one *S. epidermidis* isolate, therefore the beneficial effects of tobramycin on treatment outcomes in *S.epidermidis* could not be explained by PBT testing. It is also important to consider that four of the six *S. epidermidis* isolates were resistant to tobramycin with MIC ranging from 64 to >128 ug/ml. In this study the 6h concentration of tobramycin was 20 ug/mL and the addition to tobramycin would not inhibit resistant *S. epidermidis* isolates. When assessing cefazolin alone and cefazolin plus tobramycin regimens at 24h concentrations, the beneficial effects of tobramycin were not exhibited for most organisms since most MICs were above the 24h tobramycin concentration. (Table 7) The variations in antibiotic concentrations at 6h and 24h time points provide valuable insight on the antibacterial activity of combination antibiotics, particularly when combinations of concentration and time dependent antibiotics are administered concurrently. The role of antibiotic concentration, antibiotic sensitivity and the additional antibacterial effect of dianeal fluid in addition to its effect on antibiotic activity and organisms growth must be considered when interpreting PBT results.

In this study antibiotics were spiked into dianeal fluid at predicted 6h and 24 h concentration. At 24 h time points the addition of tobramycin did not provide additional antibacterial activity for most of the isolates. Whereas for cefazolin alone the increase in concentration at 6 h did not result in a corresponding increase in PBTs. PBT s can be explained by relating antibiotic concentration in dianeal fluid with isolate sensitivities (i.e.: MIC/ MBC). However, MIC and MBC are a measure of individual antibiotic activity in broth. Scattergrams were used to compare PBTs to calculated titres based on antibiotic concentrations (i.e.: 6h or 24h) divided by MBCs. This provided a comparison method between titres preformed in dianeal fluid and theoretical titres in broth based on MBCs.

The 6h and 24h PBTs for cefazolin alone were similar to calculated titres in broth for *E. coli* and *K.pneumoniae*. PBTs for S. *epidermidis* and *S. aureus* were higher than calculated titres indicating increased antibacterial activity in dianeal fluid. Dianeal fluid has been shown to support the growth of *S. epidermidis* and its effect on growth characteristics may have contributed to the increased antibacterial activity in dianeal fluid. Studies have demonstrated that factors such as pH and proteins can significantly alter the growth of *S. epidermidis* isolates. (Wilcox, Smith et al. 1990) Cefazolin is dependent on adequate organisms growth for antibacterial activity and variations in growth characteristics in dianeal fluid would likely affect antibiotic activity. Immune factors present in dianeal fluid may have contributed to the increase in antibacterial activity observed in dianeal fluid for some of the *S. epidermidis* and *S. aureus* isolates. Verbrugh et al observed that although *S. epidermidis, S.aureus* and *E. coli* grew well in

spent dianeal fluid factors such as peritoneal macrophages, phagocytes and peripheral blood leukocytes exhibited antibacterial activity and contributed to overall antibacterial activity. Apart from being unable to assess antibacterial activity for antibiotic combinations, MBC testing may not account for the additional antibacterial effects of dianeal fluid.

Phase 3: Pilot Study

In clinical practice, treatment selection is based on MIC results however there is little evidence available demonstrating a relationship of MICs to clinical outcome. We find this discrepancy particularly in studies examining treatment failure in pseudomonal peritonitis. Craddock et al. 1987 examined five episodes of treatment failure of pseudomonal peritonitis and found that in all cases viable strains of pseudomonas remained despite being exposed to therapeutic concentrations of gentamicin and ceftazidime. (Craddock, Edwards et al. 1987)) A pilot study was preformed to evaluate whether PBTs would be predictive of treatment outcome. Despite the small sample size and mix of pathogens and treatment regimens, a relationship of PBTs to outcome was observed at 6h and 24h concentrations. ($p=0.036$) Although further study is required these findings are very promising. PBTs are capable of measuring overall antibacterial activity in dianeal fluid and it remains feasible that PBTs could provide information on clinical outcome.

In clinical practice, dianeal fluid is sent to the Microbiology lab for Gram stain and culture. Isolates from this initial bag are usually available within approximately five

days. Prompt initiation of antibiotic therapy is crucial in the treatment of peritonitis and time to isolation would limit the use of routine PBT testing for all peritonitis cases. Difficulties would also arise in collecting spent dianeal fluid (either from the 6h or 24h bags), since in the majority of cases patients are often discharged home on intraperitoneal antibiotics. However PBTs would be beneficial in patients with more severe cases of peritonitis who are hospitalized or in patients with persistent peritonitis (i.e., > 14 days). In these patients dialysate samples could be sent to the Microbiology Laboratory for PBT testing and run against an array of different regimens so as to select the regimen with optimal activity.

Due to the small CAPD population size, clinical studies with sufficient sample sizes are difficult to conduct and treatment guidelines are often based on limited clinical data. Ideally PBTs could be used as a tool for evaluating treatment regimens for use in evaluating treatment guidelines. Despite the small sample size of the pilot study there was a strong relationship of PBTs with outcome. The median PBT for patients with resolved peritonitis (1/96 at 6h and 1/24 at 24h) was significantly greater than the median PBT for patients who failed treatment. (1/32 at 6h and ¼ at 24h) A larger sample of peritonitis cases would be required to relate PBT values to specific cure rates. Each PBT titre could potentially be associated with a specific cure rate and from this a minimum PBT for successful treatment might be determined. PBTs of new combination therapies could then be used to assess new treatment regimens prior to implementation. Ideally PBTs obtained from a large sample of the most common pathogens could be applied in empiric therapy selection prior to implementation in clinical practice. By relating PBTs to clinical cure rates and by using previously isolated peritonitis pathogens PBTs can

provide unlimited data on various antibiotic regimens, which could potentially be used in the evaluation of peritonitis treatment guidelines.

CONCLUSIONS

The purpose of this study was to develop the PBT test, which measures overall antibacterial pharmacodynamic activity for the treatment of CAPD peritonitis. PBT were characterized for the most common organisms and used to evaluate peritonitis treatment regimens. Finally, the relationship of PBTs to clinical outcome was examined.

Conclusions of this study are as follows:

1. The PBT test provides accurate and reproducible measurement of antibacterial activity within dianeal fluid. Tests are reproducible 90% of the time to an accuracy of +/- one dilution.

2. The PBT detects differences in antibacterial activity between spent dianeal fluids.

3. There is a characteristic range of PBTs for each organism at 6h and 24h concentration for cefazolin plus tobramycin and cefazolin alone regimens. *S.aureus* isolates exhibit the highest PBTs where as the lowest PBTs are seen with *P.aeruginosa* isolates. The range of PBTs the organisms correspond with published peritonitis failure rates.

4. PBTs at 6h antibiotic concentrations are greater than PBTs at 24 h antibiotic concentration, particularly for the cefazolin plus tobramycin regimen.

5. PBTs for the cefazolin plus tobramycin regimen at 6h concentration are greater than PBTs for the cefazolin alone regimen. Higher PBTs for cefazolin plus tobramycin at 6h concentration are exhibited particularly with Gram-negative organisms.

6. The pilot study demonstrates a relationship between PBTs and clinical outcome in the treatment of peritonitis.

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