PROLIFERATION OF *KLEBSIELLA PNEUMONIAE* IN MEDICATION CONTAINING VIALS FOR MULTIPLE DOSE MEDICATION AND INTRAVENOUS FLUIDS

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DECLARATION

This study represents original work by the candidate and has not been submitted any other form to UKZN or other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party. Where use was made of the work of others, it has been duly acknowledged in the text.

All routine and experimental work (growth of bacteria from storage into solid and liquid media, inoculation, determination of optical densities and colony counts), described in this dissertation was carried out by the candidate in the Department of Medical Microbiology and Infection Prevention and Control, College of Health Sciences, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa under the supervision of Professor A.W. Sturm and Professor P. Moodley.

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DEDICATION

This dissertation is dedicated to my husband, Don Misra

For always loving me and understanding me, for being my pillar of strength

PRESENTATIONS

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ACRONYMS

ATCC: American Type Culture Collection

CDC: Centre for Disease Control

g: Gram

HAI: Healthcare Associated Infection:

Hr: Hour

IPC: Infection Prevention and Control

IU: International Units

IV: Intravenous

KZN: KwaZulu-Natal

mg: Miligram

min: Minutes

mL: Mililitre

PFGE: Pulsed Field Gel Electrophoresis

µl: Microlitre

DEFINITIONS/TERMS.

Healthcare Associated Infection: Infections that occur as a result of contact with healthcare facilities. The infections are not present or incubating at the time of admission. In general they do not manifest within the first 48 hours after contact with the healthcare facility, and include infections acquired whilst within a healthcare facility but manifesting after discharge. Occupational related infection of health care workers and iatrogenic infections are also classified as HAI.

Healthcare Facility: Includes any facility in which patients are managed as well as healthy individuals that attend to obtain preventative health care. This includes hospitals, community health centres, primary healthcare clinics and emergency medical service facilities.

Key Priority Area: Based on the major concerns expressed by patients through surveys, complaints and media reports, six priority areas have been identified to fast-track improvement in provision of health care in South Africa.

Non-Fastidious Microbes: Species of microbes that are able to grow without special nutritional supplements or environmental conditions.

Pulsed Field Gel Electrophoresis: Methodology to separate of large deoxyribonucleic acid fragments by applying to a gel matrix an electric field that periodically changes direction.

Total Parenteral Nutrition: Administering fluid that contains nutrients intravenously.

PREFACE

Infection Prevention and Control is a key priority area in South Africa. Primarily, the focus is preventing healthcare associated infections. The importance of infection prevention is not just related to patient safety but it is a critical element of antimicrobial stewardship. In order to significantly reduce healthcare associated infection, it is critical to understand the mechanisms that cause these infections before implementation of measures to mitigate these risks. Monitoring compliance to policy is a key element of ensuring the desired outcome of any policy.

There are various modes of transmission which include contact, droplet, airborne, common vehicle and vector transmission. Globally and also in South Africa, there have been reports of outbreaks related to the use of medication vials. *Klebsiella pneumoniae*, Hepatitis C and even *Plasmodium falciparum* has been implicated in such outbreaks. In the most devastating outbreak reported in KwaZulu-Natal (KZN), 22 of 26 babies in a neonatal intensive care unit demised. The source was confirmed to be a vial of single use Vamin-Glucose that was used for multi-dosing. It has been postulated the multiple use of medication vials present a significant risk to the patient. The one vial, one patient advocacy presents the safest option. However, in a resource limited setting such as in KZN, cost and drug shortages are key drivers which play an important role to decide on whether or not to maintain the one vial, one patient practice. Given the circumstances faced by most healthcare facilities, it is important to determine the level of risk that different medications present by determining whether or not these can be a reservoir of a pathogen. *K. pneumoniae* is a species with low nutritional requirements and a fast multiplication rate and is often associated with healthcare associated infections and outbreaks.

ABSTRACT

Background and objectives: *Klebsiella pneumoniae* is frequently implicated in healthcare associated infections. This organism is non–fastidious and easily transmitted in a healthcare environment. Medication fluids are an ideal vehicle for transmission of these organisms. This study aimed to determine if *K. pneumoniae* would proliferate in various medication fluids.

Method: The behaviour of *K. pneumoniae* was studied in piperacillin/tazobactam, ciprofloxacin, meropenem, insulin, heparin, lignocaine, and gentamicin, concentrations (50-2 %) of dextrose, Neonatalyte® and saline. This was assessed over a 24-hour period at 25 ^{o}C and 35 ^{o}C**.**

Results*:* *K. pneumoniae* did not proliferate in the antibiotics or the multi-dose vials. The bacteria persisted in Neonatalyte® and saline. Bactericidal activity was observed in 50 %, 25 % and 12.5 % dextrose at both incubation temperatures. In 10 %, at 24 hours post inoculation, a decrease of 57 % and 50 % was observed at 25 ^{o}C and 35 ^{o}C respectively. At concentrations of 6 %, 3 % and 2 %, after 24 hours post inoculation at 25 ^{o}C, the colony count increased by 112 %, 124 % and 133 % and at 35 ^{o}C by 120 %, 134 % and 150 % respectively. There was no difference in the behaviour of *K. pneumoniae* at 25 ^{o}C and 35 ^{o}C. Neonatalyte® and saline demonstrated no increase in the cfu/mL over 24 hours.

Conclusions: Persistence of the test bacteria in saline, neonatalyte and lower concentrations of dextrose has been shown. This poses serious risk to patient care if the bacteria inadvertently enter the fluid due to infection prevention breeches.

CHAPTER: ONE

INTRODUCTION

Multiple-dose vials are liquid medication containing vials that are intended for multiple dose medication via parenteral administration. This can be by intravenous or intramuscular injection or by infusion. (1) These vials are labelled as multi-dose vials by the manufacturer and contain an antimicrobial preservative which inhibits bacterial growth. (2) The preservative is not effective against viruses, protozoan, parasites or prions. (2) Strictly, only these vials, that contain a preservative and are labelled by the manufacturer as multiple-dose vials, should be used as such.

Multiple-dose vials are intended to provide a convenient and cost effective therapeutic option. In a resource limited setting; the latter can play an important role in decision making. (3) Multiple-dose vials allow multiple aspirations. There is no wastage per dose. In any establishment, financial costs play a role in decision making but in a healthcare facility, risk to safe patient care must weigh heavily in this decision making process. In an unregulated setting, use of these vials can be detrimental to patient safety. (2)

Recommendations from the Centers for Disease Control and Prevention state that, "Multiple-dose vials should be dedicated to a single patient whenever possible. If multiple-dose vials must be used for more than one patient, it should only be kept and accessed in a dedicated medication preparation area (e.g., medication room), away from immediate patient treatment areas. This is to prevent inadvertent contamination of the vial through direct or indirect contact with potentially contaminated surfaces or equipment that could then lead to infections in subsequent patients. If a multiple-dose vial enters an immediate patient treatment area, it should be dedicated for single-patient use only." (1) The name of the patient should be on the vial as well as the date and time of first use.

The World Health Organisation (WHO) provides guidance on the safe use of multiple-dose vials. (4) Whilst these recommendations are applicable to vaccines, the scientific rationale applied by WHO are also relevant to other multiple-dose vials. In order to ensure the safety and efficacy of the vial, there are various factors that must be taken into consideration. These include assessing the preservative to determine effectiveness in preventing bacterial contamination and also stability of the medication over various temperatures and times. WHO further advises that, if the medication does not contain preservatives or if there is insufficient evidence that the preservative is effective in preventing bacterial contamination, a time limit of six hours or one immunization round should be applied for use of the vial. (4)

Thiomersal is a preservative used in DTaP and one DTaP-Hib combination vaccine. (5) It is a compound that contains mercury, which is found naturally in the environment. (5, 6) In 1999, Food and Drug Administration (FDA) conducted a review of the use of thiomersal in childhood vaccines and found no evidence of harm from its use as a vaccine preservative, other than a reaction at the injection site. In 2001, the Institute of Medicine's Immunization Safety Review Committee reached a similar conclusion, based on a review of available data, and again in 2004, after reviewing studies performed after its 2001 report. (6)

The National Department of Health: South Africa provides guidance on the use of multiple-dose vials. This, however, pertains largely to vaccines and is aligned with the WHO recommendations. (7)
The South African Society of Anaesthesiologists (SASA) Guidelines for Infection Control in Anaesthesia in South Africa, 2014, gives vague guidance on the safe use of multiple-dose vials. This document fails to give a precise definition of multiple-vials, exact disinfection procedure, importance of adherence to manufacturer's instructions, criteria for use on multiple patients, disposal of the vial or storage limitations once opened. (8) A significant number of medications used in anaesthesia are intended for multiple dosing. (9, 10) In a surveillance of anaesthesia medication by Heever *et al* (2012), a microbial contamination was found in 6 % was found. (9)

The Department of Health: KwaZulu-Natal (KZN), has formulated a policy entitled: "Correct use of single-dose and multiple-dose injections and large parenteral and total parenteral nutrition solutions in order to prevent contamination", (2005). (10) This policy allows multiple-dose vials to be used for multiple withdrawals provided that infection prevention measures are adhered to, the vial has not been submerged in water, the date of opening is indicated, room air is not injected into the vial and that the storage time once opened is 30 days or as per manufacturers' recommendation. (10) Vials used in this way must be discarded thereafter. (10) The policy however recommends storage at 4 ^{o}C. (10) Preservatives exert their action on organisms that are actively metabolising. At lower temperatures, metabolism of most bacteria is reduced. Refrigeration of medications at lower temperatures when not indicated by the manufacturer can have an opposite effect as the bacteria may then be protected from the action of the preservative. When injected into the patient, the preservative is then diluted and the bacteria enter a nutrient rich environment which allows active bacterial proliferation. (11, 12)

The Department of Health KZN: Infection Prevention and Control Guidelines, Chapter 24: In neonatal intensive care units it is recommended that the vial should be labelled with date and time of opening and stored in accordance with the manufacturers' recommendation. (13) The vial may be used for multiple patients within the same medication round. (13) The medication round should not exceed twenty minutes. (13)The KZN Infection Prevention and Control Guideline recommends

cleaning of the vial with 0.5 % chlorhexidine in 70 % alcohol solution and allowing the rubber septum to dry before use. (13) Single use of needles and syringes is also recommended. (13)

Whilst guidelines are in place to guide the safe use of multi-dose vials, factors such as infrastructure, staffing and overcrowding contribute to behavioral changes that have negative impact on infection prevention practices. (14) Furthermore financial costs (15) and drug shortages often dictate a more cost centered approach to the use of medication fluids. In the absence of evidence based regulations and monitoring of the use of medication vials, the consequence is possibly detrimental to patient care and subsequent medical costs

Klebsiella pneumoniae is a gram negative bacterium (16) which is frequently implicated in healthcare associated infections (HAI) and hospital outbreaks (17–19) The bacterium is spread via contact transmission. (16) *K. pneumoniae* is part of the commensal flora of the intestinal tract. (16) The organism is non-fastidious and easily transmissible in a healthcare environment. (16) Patients admitted to high risk areas in a healthcare facility such as Intensive Care Units and Neonatal Units are vulnerable to acquiring infections due to the invasive nature of procedures in these units and indwelling devices. (16) The use of parenteral medication is seen as a major contributing risk factor to HAIs.

CHAPTER TWO

BACKGROUND

In 2005, 22 out of 26 (86 %) babies demised during one of the most devastating outbreaks in KwaZulu-Natal (KZN). The outbreak occurred in Mahatma Gandhi Memorial Hospital. *Klebsiella pneumoniae* was isolated from the blood cultures of 25 babies. One baby had *Klebsiella oxytoca* in the blood culture. Various environmental sites, formula feeds, expressed breast milk, medication and intravenous fluids were all tested for *K. pneumoniae.* All were found to have no growth besides four bottles of Vamin-Glucose. The unopened bottles were also tested and found to be sterile. The Vamin-Glucose was for single use but in this case it was used as multiple-dose medication. (20) Two years after this outbreak, *K. pneumoniae* was once again implicated in an outbreak at Prince Mshiyeni Regional Hospital. Five out of seven (71 %) babies demised. Re-use of intravenous fluids and medications were once again implicated. (21)(22) After this outbreak, multiple dosing was discontinued. (23) The KZN: Department of Infection Prevention and Control adopted the Centre for Disease Control's "One and Only Campaign" approach. This stipulated the use of one needle, one syringe and one medication vial per patient, even for multiple-dose vials. (24) Whilst this may have been the safest option, the one vial per patient approach was not sustainable. Thus, it became necessary to study the proliferation of *K. pneumoniae* in the medication fluids and the parameters that could contribute to contamination of medication fluids commonly used in a Regional Hospital in KZN.

Parenteral administration of medication forms one of the cornerstones of clinical care. (25) Several medications are available in multiple-dose vials. Reasons for medications being presented as multiple-doses per unit include convenience to the user and cost effectiveness. Those vials labelled as multiple-dose vials by the manufacturer are designed for multiple use. These vials contain a preservative to inhibit bacterial growth (25) this does not protect the fluid from fungal or viral contamination. (26) Multiple-dose vials are subjected to many withdrawals under the assumption that the vials are sterile and resistant to contamination. The rubber stoppers of such vials have a self-sealing capacity and are able to withstand multiple withdrawals. Self-sealing capacity refers to the ability of the rubber stopper to prevent microbial entry into the vial and prevent product loss during and between uses. (27)

Single dose medications are intended to be used for a single injection on a single patient. These medications are usually labelled as single dose vials and lack any antibacterial agents that could prevent bacterial growth. Some single dose vials may appear to contain multiple doses due to the volume they contain. In these instances, healthcare professionals are often tempted to use these for multiple dosing to avoid wastage. Such decisions have compromised patient safety and consequently escalated hospital costs.

2.1 Outbreaks

2.1.1 Insulin identified as source of outbreak

Contaminated medication fluids have been implicated in outbreaks. (15, 20, 28–35) Some were confirmed microbiologically but most were hypothesized after epidemiological investigations. In 2001, in an intensive care unit in Malaysia, 12 patients had blood stream infections with *Serratia marcescens*. (36) Environmental sampling revealed insulin and a sedative solution to be the source. The strain relatedness was confirmed using pulsed field gel electrophoresis (PFGE). The authors did not mention if the vial was used as per manufacturer's recommendation. (36) In a publication from Italy in 2006, another outbreak with *S. marcescens* was reported in a surgical ward in a community hospital. (37) Six patients had positive blood cultures with this organism. Five patients received total parenteral nutrition (TPN) but all six received intravenous insulin. Insulin was diluted with 10 mL of poligeline. This was then added to the TPN solution in order to decrease adhesion of insulin to the bag. The poligeline was from a 500 mL bottle which was meant for single-use but was used for more than one patient and stored at room temperature for three to seven days. *S. marcescens* was cultured from the in-use TPN solutions. The in-use insulin and poligeline was not cultured as the vials were discarded. The authors concluded that the most probable sources was the TPN solution which insulin was added with/or poligeline solution. (38)

2.1.2 Heparin identified as source of outbreak

2.1.2.1 Heparinised saline syringe contamination at manufacture plant

In a multistate outbreak in the Unites States of America, *Pseudomonas fluorescens* was isolated from various specimens including blood obtained from indwelling central venous catheters and peripheral veins; sections of explanted catheter and from a catheter wound site. (31) Eighty patients were involved. This outbreak lasted from December 2004 to March 2006. Common to all of these patients was the use of heparinised saline intravenous flushes. The syringes were prepared at a compounding pharmacy. A compounding pharmacy is a pharmacy where medications are altered or mixed to create customised medications. Nine lots of prepared syringes were tested. Seven were found to be positive for *P. fluorescens.* The isolates from the syringes and the fifty-nine patients were then subjected to PFGE. The isolates were found to be genetically related. The exact constituent of the syringes and the manner in which the heparin was used and stored was not mentioned. (31) In another multistate outbreak, this time with 162 cases over nine states in the Unites States of America, *S. marcescens* was involved. (39) The outbreak occurred from November 2007 to January 2008. A major risk factor identified was that all patients received heparin flushes from a particular lot of prefilled syringes. The heparin from the unopened syringes was cultured and grew *S. marcescens.* These isolates were found to be genetically related to the patients' isolates. (39) More recently, in 2018, heparin saline syringes

were once again responsible for an outbreak of *S. marcescens* involving 14 children. The children received intravenous medication via a catheter or central line. *S. marcescens* was not isolated form the unopened syringes but all syringes were recalled out of caution. (40)

2.1.2.2. Heparin catheter lock solution contamination

Central venous catheters are used for prolonged periods of time. They are used for administration of medication, blood products, TPN, for haemodialysis and to monitor the haemodynamic status of patients. In 2003, in San Paulo, Brazil, 32 cases of *Pseudomonas putida* and nine cases of *Stenotrophomonas maltophilia* were reported. (41) Five of the 32 cases of *P. putida* with were mixed with *S. maltophilia*. All patients had central venous catheters. The catheters were infused with a heparin catheter lock solution. *P. putida* was isolated from the prefilled syringes. *S maltophilia* was not cultured from the syringes but the strains showed relatedness to each other. Genotyping confirmed a link between the *P. putida* from the patients and those from the syringes. The authors highlighted the risk associated with the use of intravenous fluids prepared by a compounding pharmacy. (41)

2.1.2.3 Multiple-dose vial of heparin identified as source of outbreak

In 2008, in Hospital de Clinicas in San Paulo, Brazil, 24 bacteraemia cases were investigated as part of a possible outbreak. (33) The organisms involved belonged to the *Bukholderia cepacia* complex. Twenty-three isolates from 21 patients could be retrieved for genotyping. One isolate did not belong to the *B. cepacia* complex. The remainder isolates were found to be polyclonal with *B. multivorans* as the predominant type. Two different isolates from two patients were typed and these were not genetically related. The patients with the predominant type had their central venous catheters flushed with saline and heparin. The multiple-dose vials were not available for culturing. The link between the multiple-dose vials and the bacteraemias was hypothesised after the interrogation of patient details and an audit of infection prevention measures. It was found that there were many breaches in compliance with infection prevention protocols. This included poor hand hygiene practices, poor environmental hygiene, poor handling of medication vials and medication fridges were not temperature monitored. Laminar flow cabinets used to store pre-prepared syringes until administration were poorly maintained. Authors hypothesised that the laminar flow cabinet was also a probable source but cultures were negative. This negative culture was attributed to not dismantling the laminar flow properly for swabbing. Following re-enforcement of good infection prevention measures and proper maintenance of the laminar flow cabinet including cleaning and disinfection, the outbreak was halted. (33)

2.1.3. Lidocaine identified as source of outbreak

In August 2001, in Tennessee, United States of America, five patients were investigated for septic arthritis or soft tissue infection. (12) *Staphylococcus aureus* was isolated. (12) Pulsed Field Gel Electrophoresis (PFGE) was done on these isolates. Four were found to be related. Common amongst these patients was that they received lidocaine injections between August 13th and August 17th. Unopened vials were tested and found to have no growth. It was reported that the in-use vial of lidocaine was stored at 4 ^{o}C but the vial was not available for testing. Although the lidocaine had a preservative, the preservative could only exert its action on actively metabolising organisms. In the refrigerator, most organisms slow down their metabolism or do not metabolise at all. Thus refrigeration of the vials actually protected the organisms from the action of the preservative which later caused an infection when injected into the patient. (11, 12) Since all patients had received lidocaine injections from the same vial, it was hypothesised that this was the source. (12)

2.1.4. Multi-dosing from single-use dextrose identified as source of outbreak

In 1996, in a neonatal intensive care unit in Puerto Rico, six patients were diagnosed with blood stream infection. *Enterobacter cloacae* and *Pseudomonas aeruginosa* were isolated from five patients and one had *E. cloacae* only. (42) The *E. cloacae* isolates belonged to one clone and so did the *P. aeruginosa* isolates. An audit of the infection prevention practice revealed non-compliance with many protocols. Apart from poor hand hygiene it was found that the medication room was cluttered with used utensils and equipment, prepared syringes were found amidst pools of stagnant water, and rubber septa of medication vials were disinfected with povidone-iodine but not allowed to dry before use. Intravenous fluids and electrolyte solutions were prepared by the staff in the neonatal intensive care unit. The intravenous solution was known as Mezclas and consisted of calcium gluconate, potassium chloride, saline and 5-20 % dextrose. The dextrose was added to the solution from a 50 mL vial that was used until empty. Environmental sampling was done. The cultures from the swabs taken from the hand wash facility grew *E. cloacae* and *P. aeruginosa*. These were unrelated to the outbreak strains. The dextrose could not be microbiologically confirmed, as the source of the outbreak as the in-use vial of dextrose was discarded. Authors concluded that the *E. cloacae* and *P. aeruginosa* had been brought in by a patient and transferred onto the hands of the healthcare workers. Subsequent non-compliance to hand hygiene and disinfection of the rubber septum contaminated the rubber septum. The organisms were then inadvertently introduced into the fluid during withdrawal of the fluid. (42)

2.1.5. Multiple-dosing of single-use Vamin-Glucose identified as source of outbreak

In 1996, an outbreak occurred in a provincial hospital in Gauteng. There was an outbreak of necrotising entercolitis and nine babies demised. The causative organism was *E. cloacae,* which was isolated from three blood cultures. The unit was short staffed and poor hand hygiene was observed. Vamin-Glucose was added to saline or neolyte. Although meant for single dosing, the Vamin-Glucose was multiple dosed and visibly turbid. Environmental sampling was done. The outbreak strain of *E. cloacae* was isolated from the hands of the healthcare workers, nebuliser water, neolyte, saline and the Vamin-Glucose. The authors concluded that the Vamin-Glucose was the source and highlighted the importance of maintaining basic infection prevention measures. (34)

2.2 Contamination of in-use medication

In Wisconsin, United States of America, Sheth, *et al* tested 197 in-use medication vials. (43) Prior to testing, there had been between one and ten withdrawals from each vial. All vials were tested for bacterial and fungal contamination. There was no growth after seven days of incubation. (43) In another study, in Namazi Hospital, Shiraz, Iran, in 2006, six hundred and thirty six in-use medication vials were collected from thirty six wards. The medications were mainly potassium chloride, saline, sodium bicarbonate, calcium carbonate, insulin, and distilled water. None of the vials were used beyond expiry date but all were used for multiple patients and all were stored at room temperature. The bacterial contamination was detected in 5.6 % of these vials. The most frequently isolated organism was *S. epidermidis*. The only vial of insulin tested was from the emergency unit and this contained beta haemolytic streptococci. *S. epidermidis* and *Streptococcus viridans* were isolated from the saline. (30) Insulin was found to be the most frequently contaminated fluid in a study by Baniasadi *et al* (2013). Two hundred and five vials were tested. Eleven were contaminated. Three vials of insulin were positive either with *Candida species*, *Micrococcus species* or *Escherichia coli*. These vials were stored between 4-6 ^{o}C. (44) This study highlighted the impact on insulin as a result of non-adherence to manufacturers' recommendations on storage temperature.

Longfield *et al* tested 1223 vials including insulin, heparin and xylocaine. None of the vials were contaminated. (32) In two separate studies, in-use vials with heparin were tested for bacterial contamination. Mattner *et al* tested 41 vials of heparin which represented 18 % of the total vials tested. (29) Baniasadi *et al* tested 11 vials of heparin which represented 5 % of the total vials tested. (44) Both the studies did not find any bacterial growth from these vials.

Rathod *et al* aimed to assess the sterility and stability of insulin used in the community. (45) Sixty nine vials from 50 patients who used insulin at home were tested. The average duration of use was 55 days, which exceeded the recommended 28 days. All patients used disposable syringes but nine admitted to re-using these between two and fourteen times. Eight of the vials grew 1 cfu/mL of either

S. epidermidis or *Propionibacterium acnes*. One patient was found to have an injection site abscess with *K. oxytoca.* This study concluded that even when insulin was used for beyond 30 days; it did not get contaminated with pathogens. (45)

Khalili *et al*, collected in-use multiple-dose vials, single dose vials and admixtures. (46) These were then tested for bacterial contamination. Included in this collection were three multiple-dose vials, 40 single dose vial and 109 admixtures. The admixtures were prepared either in the treatment room or a clean room. Ninety two admixtures were prepared in the treatment room and 17 were prepared in the clean room. The clean room was defined as an area which was used solely for preparation of medication. The room was equipped with a laminar flow cabinet, air filter and users were required to wear specialised clothing. (46) Of the 40 single dose vials, four vials were 50 % dextrose. Of the 108 admixtures, there were 45 admixtures with 5 % dextrose, seven admixtures with 10 % dextrose, eight admixtures with 5 % dextrose and 0.9 % saline and three admixtures with 0.33 % dextrose with 0.3 % saline. Only one of the total of 92 admixtures prepared in the treatment room (1 L NaCl 0.45 % plus 10 mL KCl 15 % and 2.5 mL $MgSO_4$) was contaminated with *Bacillus subtilis.* (46) Authors concluded that although the multiple-dose vials did not yield any growth, the manner in which admixtures were prepared was a major risk. The importance of hand hygiene, injection safety, and environmental hygiene, preparation of medication in a clean room and correct handling of medication vials were listed as critical to prevent bacterial contamination of intravenous fluids. (46)

In 2001, a study was carried out in Hanover, Germany to determine the risk associated with the use of multiple-dose vials. (29) All opened vials were collected from the wards. The vials were tested for bacterial contamination and checked for storage conditions and compliance to manufacturers' instructions. Two hundred and twenty-seven vials were collected. One hundred and thirteen of the vials were undated. Of the one hundred and fourteen dated vials, fifteen had passed the expiration date by up to fourteen days. *S. epidermidis* was isolated from saline and from a spike device on another saline bottle. The bacterial contamination rate was found to be 0.9 %. It was concluded that preservative free solutions such as saline could possibly lead to severe infections and that although *S. epidermidis* was isolated, it was conceivable that any organism can contaminate these fluids. Vial spike devices that contained filters to prevent bacterial contamination were used. However, this study showed that even these devices were prone to contamination. The authors felt that use of these filter containing devices could lead to a false sense of safety and thus encourage improper handling of vials. They recommended hand hygiene and proper handling of medication fluids but the most important recommendation was to avoid multiple uses of preservative free solutions. (29)

2.3 In vitro studies

Highsmith, *et al* experimented with various multiple-dose vials in order to assess if the medication fluids were either bactericidal, allowed persistence or proliferation of bacteria. (47) Amongst the medication fluids tested were insulin, heparin and lignocaine. Insulin was tested at 4 ^{o}C and 25 ^{o}C. Thirteen micro-organisms were included in this panel: *Acinetobacter calcoaceticus, Enterococcus, Enterobacter agglomerans, E. cloacae, Escherichia coli, K. pneumoniae, Proteus mirabilis, P. aeruginosa, Pseudomonas cepacia, S. marcescens, S. aureus, S. epidermidis,* and *Candida albicans.* A single colony of each organism was picked up and inoculated into brain heart infusion broth. The broth was then incubated for 18 hours at 35 $\pm$ 1 ^{o}C. The cells were washed three times using 0.25 % peptone water in saline. Cells were then resuspended in 0.25 % peptone water in saline. The resuspended cells were the diluted to achieve an inoculum density of 300 cfu/mL and then inoculated into 30 mL of the medication fluid to yield a concentration of 10 cfu/mL. The experiment was repeated using 0.9 % saline to wash, resuspend and dilute. The experiment was carried out up to 168 hours. With insulin, when the inoculum was prepared with 0.25 % peptone water in saline or 0.9 % saline, *K. pneumoniae* did not survive at either of the incubation temperatures. *C. albicans* survived at 168 hours whilst *Enterococcus* showed proliferation at 25 ^{o}C. The insulin was retested with *Enterococcus* but with an inoculum prepared with 0.9 % saline. Authors declared these results to be inconclusive but the number of organisms at 168 hours were less that the initial inoculum. (47) *K. pneumoniae* survived in heparin up to 24 hours only. The lignocaine showed proliferation of most of the test organisms, including *K. pneumoniae*, when the inoculum was prepared with 0.25 % peptone in saline. When retested with 0.9 % saline, lignocaine was found to be bactericidal to *K. pneumoniae* by 24 hours post inoculation. This study had two significant findings. Firstly, that a small amount of peptone added to pharmaceuticals was sufficient to allow survival or proliferation of bacteria and yeasts. It was believed that the peptone either inactivated the preservative or provided sufficient nutrients to allow bacterial growth. The second finding was that bactericidal activity was higher at 25 ^{o}C than 4 ^{o}C. (47)

Longfield, *et al* tested behaviour of *C. freundii, E. aerogenes, E. agglomerans, Flavobacter meningosepticum, K. pneumoniae, P. cepacia, S. maltophilia, S. marcescens, S. aureus* and *S. epidermidis* in medication in multiple-dose vials. (48) This included insulin, heparin and lignocaine. An inoculum of 1000 cfu/mL in saline was used. (48) All experiments were done at 4 ^{o}C and 22 ^{o}C. The mean time to sterility in insulin was 5.4 hours at 22 ^{o}C and 25 hours at 4 ^{o}C, heparin was 5.3 hours at 22 ^{o}C and 33.4 hours at 4 ^{o}C and lignocaine was 15.5 hours at 22 ^{o}C and 132 hours at 4 ^{o}C. The study showed that whether or not bacteria allow persistence or proliferation depended on the type of medication, preservative used, storage temperature and specific risks. Thus, when developing guidelines for use of multiple-dose vials, it should be done on product to product basis. (48)

Rathod *et al*, 1985 tested insulin with *P. aeruginosa* and *S. aureus* at 22 ^{o}C and 4 ^{o}C. (45) Inoculum densities of 10^2, 10^4 and 10^6 cfu/mL were used. Both organisms were eradicated in 24 hours at 22 ^{o}C with all inoculum densities. The bacterial killing was much slower at 4 ^{o}C. This took 10 days for *P. aeruginosa* with an inoculum of 10^6 cfu/mL and with *S. aureus* persistence was demonstrated up to 17 days. This study showed that insulin does not get contaminated with pathogens even if the vial was used for > 28 days and that the preservative was more effective at 22 ^{o}C than 4 ^{o}C. (45)

Studies investigating contamination of in-use medication have shown that bacterial contamination of medication fluids can occur (29, 43, 46, 49) whilst sterility studies showed that the fluids can resist bacterial growth (44, 47, 48) but, as evidenced by the outbreaks, medication vials have been identified as microbial reservoirs. (20, 33–35) Healthcare associated infections adversely affect patient safety and also have negative impact on costs and resources. Guidelines on safe use of medication vials should be evidence based.

CHAPTER THREE

AIMS AND OBJECTIVES

3.1 Aim

To determine the possibility of *Klebsiella pneumoniae* ATCC 700603 proliferating in multiple-dose medication and intravenous fluids

3.2 Objectives

1. To determine the growth curve of *K. pneumoniae* in brain-heart infusion broth
2. To conduct a microbiological challenge test using *K. pneumoniae* of
    a. vials and ampoules containing medication used for multiple-dose intravenous administration
    b. intravenous fluids
3. To determine the minimum number of colony forming units (cfu) of *K. pneumoniae* that results in contamination of medication and intravenous fluids
4. To determine the concentration of dextrose that allows multiplication of *K. pneumoniae*

CHAPTER FOUR

MATERIALS AND METHODS

4.1 Ethics Approval

This research project received approval from the Biomedical Research Committee at the University of KwaZulu-Natal. Ethics number: BE 222/13. This research project did not involve collection of specimens from patients, healthcare establishment environment or healthcare workers.

4.2 Study material

Multiple-dose medication vials and ampoules as well as intravenous fluids commonly used at the King Edward VIII Hospital were used in this investigation. King Edward VIII hospital is the second largest hospital in the Southern hemisphere, providing regional and tertiary services to the whole of KZN and Eastern Cape. It has 922 beds and sees +/- 360 000 out-patients. The hospital is situated in the eThekwini District. (50) For all tests *Klebsiella pneumoniae* ATCC 700603 was used.

4.2.1 Vials.

The medication in vials was accessible by piercing a hypodermic needle through a rubber septum. The vials tested and their contents included Tazobax®(Aspen, Johannesburg, South Africa) 20 mL with 4g piperacillin and 0.50 g tazobactam as sodium salts (51), Ciprocina®(Claris Life sciences, United Kingdom Limited, Cheshire, United Kingdom) 100 mL ciprofloxacin 2mg /mL (52), Meronem®(AstraZeneca Pharmaceutical, Bryanston, South Africa) meropenem 500 mg in 20 mL (53), Actrapid HM(ge)®(Novo Nordisk, Sandton, South Africa) with 100 units/mL genetically engineered mono-component neutral insulin, preserved with 0.3 % m-cresol (54), Heparin Sodium® (Fresenius Kabi, Midrand, South Africa) with 25000 i.u/mL heparin preserved with chlorocresol 0.1 % w/v (55) and Lignocaine HCL®-(Fresenius Kabi, Midrand, South Africa) 2 % (w/v) preserved with methyl hydroxybenzoate 0.1 % w/v.

4.2.2 Ampoules

Ampoules tested included Gentamicin (Aspen, Johannesburg, South Africa) 80 mg/2 mL and Dextrose 50 %(Fresenius Kabi, Midrand, South Africa)(20 mL).

4.2.3 Intravenous Fluids

Two intravenous fluids were tested. These included Neonatalyte®(Adcock Ingram, Midrand, South Africa) which comprised of Glucose Monohydrate 110 g/L, Potassium Chloride 1.12 g, Calcium Chloride Dihydrate 367 mg, Magnesium Chloride Hexahydrate 102 mg, Sodium Lactate 2.0 g and Phosphoric Acid 367 mg and Sodium chloride, 0.9 % (Adcock Ingram, Midrand, South Africa).

4.3 Method

4.3.1 Growth curve

The growth curve of *K. pneumoniae* ATCC 700603 was determined at:

- 25 ^{o}C, which is the median temperature to be maintained in Neonatal Intensive Care and nursing units. The temperature in such units must be maintained at 24-26 ^{o}C (Department of Health: KZN Infection Prevention and Control Guidelines. (56–58)
- 35 ^{o}C, the optimum temperature for growth of *K. pneumoniae.* (59)

K. pneumoniae was stored in cryovials (Corning, Globe Scientific, Mahwah, Unites States of America) at -80 ^{o} C (Nuaire Glacier , Model No NU 9483E, Plymouth, Unites States of America) in the repository at the Department of Medical Microbiology/Infection Prevention and Control. For storage, overnight cultures of *K. pneumoniae* were picked off the agar plate and inoculated into the cryovials containing glass beads and a storage broth which comprised of brain heart infusion (Oxoid LTD, Basingstoke, Hampshire, England) and glycerol (Merck Chemicals, (Pty) Ltd, Germiston, South Africa). (Appendix C.3) The vials were then gently shaken, allowing the bacteria to coat the glass beads. The excess broth was then aspirated and discarded and the vial frozen.

Following thawing of the contents of a cryovial at room temperature, one glass bead was aseptically removed and rolled onto a Columbia Blood Agar plate (Oxoid LTD, Basingstoke, Hampshire, England) with 5 % horse blood (Appendix C.2). Following inoculation, the plates were incubated overnight, aerobically at 35 ^{o}C.

4.3.1.1 Inoculum Preparation / Growth Curve

The next day, one colony was touched with a sterile straight wire and inoculated in 5 mL of brain heart infusion broth. (Appendix C.5) The broth was then incubated overnight, under aerobic conditions at 35 ^{o}C. Of this overnight culture, 100 µL was inoculated into a 50 mL polypropylene tube (Greiner Bio-One GmgH, Frickenhausen, Germany) containing 45 mL of brain heart infusion broth. (Oxoid LTD, Basingstoke, Hampshire, England). The tube was then inverted to mix the culture.

To determine the optical density, two cuvettes (Kartell Labware, Usmate, Italy) were used. One containing 2 mL of sterile brain heart infusion broth was used as a blank, the other as test cuvette. The test cuvette was filled with 1800 µL of brain heart infusion broth and 200 µL of the freshly inoculated suspension in brain heart infusion broth. The optical density was obtained at a wavelength of 600 nm using the Biochrom, Lightwave 11 Spectrophotometer, Model 80-3003-72, Cambridge, United States. . Readings were done in triplicate and the average thereof was taken as the final reading. Readings were taken every half an hour up to ten hours.

Incubation was under aerobic conditions at 25 ^{o}C or 35 ^{o}C for the length of the experiment. Incubation commenced after the first readings were taken. Sterile, 2 mL, microcentrifuge tubes (Eppendorf, Hamburg, Germany) were used. Nine hundred µL of phosphate buffered saline (Oxoid LTD, Basingstoke, Hampshire, England) (Appendix C.4) was inoculated into each tube. One hundred µL of the brain heart infusion culture was then inoculated into the 900µL of phosphate buffered saline to give a dilution factor of 1:10.

Ten serial 1:10 dilutions were then performed. This was done by aspirating 100 µL from the first tube and inoculating into the next. The tube was then closed and vortexed for 30 seconds.

(Velp®Scientifica, Model No/code F202A0230, Usmate, Italy) One hundred µL from this tube was then aspirated and inoculated into the next tube. This was then continued until the last tube was reached. One hundred µL from each dilution was then inoculated onto a labelled MacConkey plate (Oxoid Ltd, Basinstoke, and Hampshire, England) (Appendix C.1). The inoculum was spread using disposable loops (Sterilin, Scientific Laboratory Supplies Ltd, Scotland, United Kingdom). The plates were incubated overnight, under aerobic conditions at 35 ^{o}C.

The next morning, the plates were read to count the colonies. Plates with a number of colonies between 20 and 200 (60) were counted. This value was then corrected for the dilution factor to determine the actual number of cfu at each time point. The experiment was done in triplicate at both incubation temperatures.

4.3.2 Microbiological challenge test

4.3.2.1 Preparation of the inoculum

K. pneumoniae was inoculated onto MacConkey Agar and subjected to the four way streak method to obtain single colonies. (61) The plates were then incubated overnight, aerobically at 35 ^{o}C. The next morning, one colony was touched and inoculated into 5 mL of brain heart infusion broth. The tube was incubated aerobically at 35 ^{o}C up to an OD of 1.5. The optical density was determined at a wavelength of 600 nm using a spectrophotometer. An OD of 1.5 yielded approximately $1.2x10^{9}$ cfu/mL. The culture was then washed using phosphate buffered saline in order to remove all traces of

the broth as the broth could provide nutrients necessary to promote the growth of the organism. This was done three times at 4400 rpm for 3 minutes per spin. (Eppendorf centrifuge, Model No 570207910, Hamburg, Germany). After the final spin, the phosphate buffered saline was decanted into a bucket containing disinfectant. The pellet was then re-suspended in 5 mL of phosphate buffered saline. Five serial 1:10 dilutions were then performed to yield approximately 1.2-1 x 10^4 cfu/mL. One hundred µL was used thus deriving and inoculum density of approximately 1.2-1 x 10^3 cfu. (62)

Before the actual simulated contamination of the test vials, it was first established that the fluid in the vials was not contaminated. This was confirmed by culture. The vial rubber septa and intravenous ports were swabbed with 70 % alcohol and allowed to dry. A sterile 2 mL syringe (Avacare, Supra Healthcare, Johannesburg, South Africa) with a 26G x1 needle (duraSurge, Omnisurge, Johannesburg, South Africa) was used for each vial and intravenous fluid bag. Five hundred µL from the vials and 1 mL from the intravenous fluid bags were aspirated and inoculated onto MacConkey plates. The plates were incubated overnight, aerobically at 35 ^oC.

One hundred µL of the *K. pneumoniae* suspension was dispensed on each vial rubber septum and on the rubber septa of the bag ports. The septa were allowed to dry. Vials and bags were sampled with a different sterile syringe without disinfection: every half an hour for eight hours on the day of contamination and 24 hours post inoculation. At each time point 500 µL was aspirated from each vial and 1mL from each bag. The aspirates were inoculated onto MacConkey plates. These plates were incubated overnight, under aerobic conditions at 35 ^oC. The next morning, colonies were counted. Tests were done in triplicate.

The ampoules containing 50 % dextrose ampoule and gentamicin were tested differently and also conducted in triplicate. One hundred µL of the *K. pneumoniae* suspension was dispensed on a gloved thumb and spread using the tip of the pipette (Gilson, Middleton, United States of America). The ampoules were then opened using this thumb. The ampoules were left open and sampled at same time points as for vials and bags. Five hundred µL was aspirated from the dextrose and hundred µL from the gentamicin ampoules. The aspirates were inoculated onto MacConkey plates. These plates were then incubated overnight, under aerobic conditions at 35 ^oC, after which colonies were counted.

At the end of the last sampling, an impression culture was done on MacConkey agar. Figure 1 shows the appearance of the rubber septa after the sampling episode. The rubber septa of the vials and the ports of the bags were pressed onto the agar plate. The plates were incubated overnight, aerobically at 35 ^oC after which colonies were counted.

Figure 1: Appearance of the vial septum after sampling episodes

4.3.3 Minimum inoculum for growth in different medication fluids

To define the minimum number of cfu of *K. pneumoniae* that results in contamination of insulin heparin, lignocaine, Neonatalyte® and saline.

A bacterial suspension prepared as described in 4.3.2.1 was then diluted to yield approximately 20000 cfu/mL, followed by five two-fold serial dilutions, resulting in test inocula of 20000, 10000, 5000, 2500, 1250 and 625 cfu/mL. Of each of the test fluids, 1900 µL was pipetted in six well of a 24-well tissue culture plate. Hundred µL of each test inoculum was added to one of these wells. This was done twice in triplicate. One set of plates was incubated at 25 ^{o}C and one at 35 ^{o}C. Figure 2 and 3 depicts the set up experiments.

To measure increase in number of bacteria during incubation, 100 µL of each well was flooded onto a MacConkey agar immediate after inoculation, after 0.5 hours and every hour for 8 hours. The last sampling was after 16 hours. These MacConkey agars were incubated overnight, aerobically at 35 ^{o}C. The next day, the numbers of colonies were counted.

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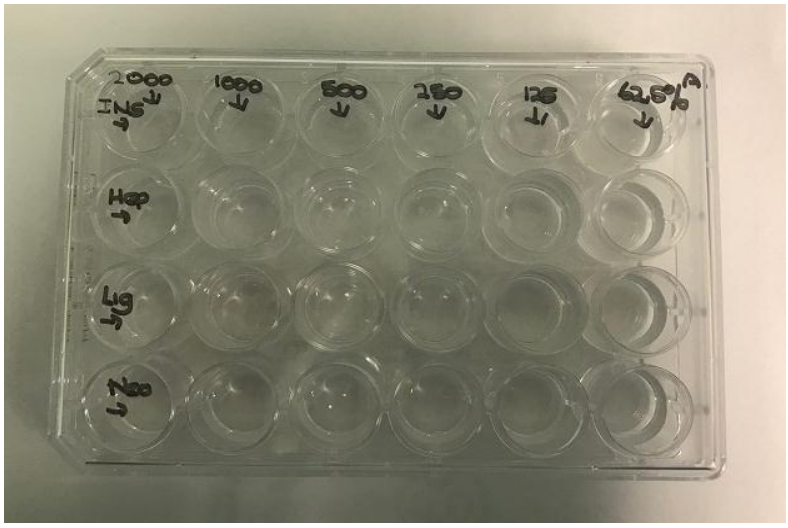

Figure 2: 24 well tissue culture plate with *K. pneumoniae* inocula in medication fluids

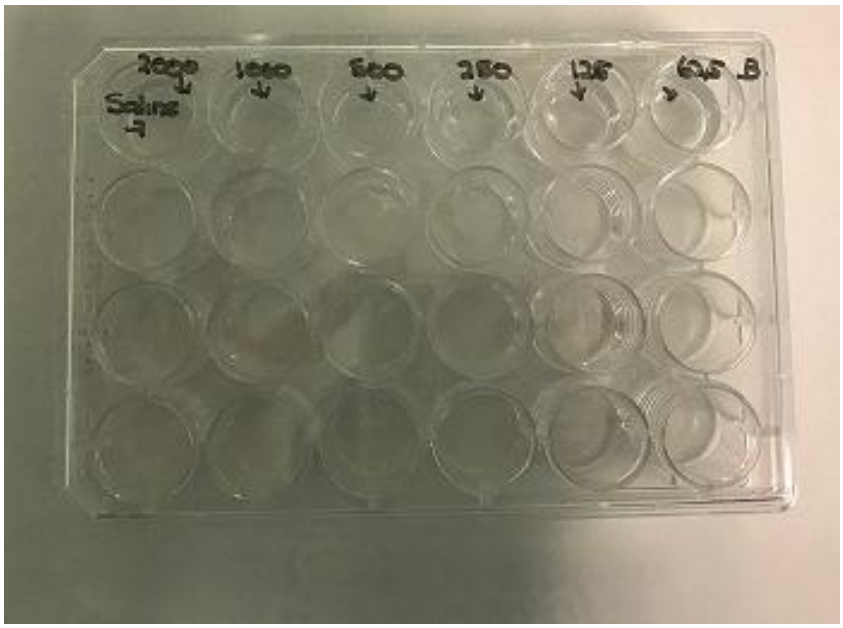

Figure 3: Illustration of a 24 well tissue culture plate with *K. pneumoniae* inocula in saline

4.3.4 Effect of dextrose concentration on growth of K. pneumoniae

To determine the effect of various concentrations of dextrose on the growth of *K. pneumoniae.*

For the first experiment (experiment A), four two-fold serial dilutions of 50 % dextrose were made in 24 well tissue culture plates using sterile distilled water as diluent. Final concentrations were 50 %, 25 %, 12.5 % and 6.25 % in 1900 µL volumes. The 6 wells of each row contained the same dextrose concentration (Figure 4)

For the second experiment (experiment B), 1900 µL of 10 % dextrose solution in distilled water was dispensed in the wells of the first row of a 24-well tissue culture plate, 1900 µL of 3 % in the second row and of 1900 µL 2 % in the third row (Figure 5).

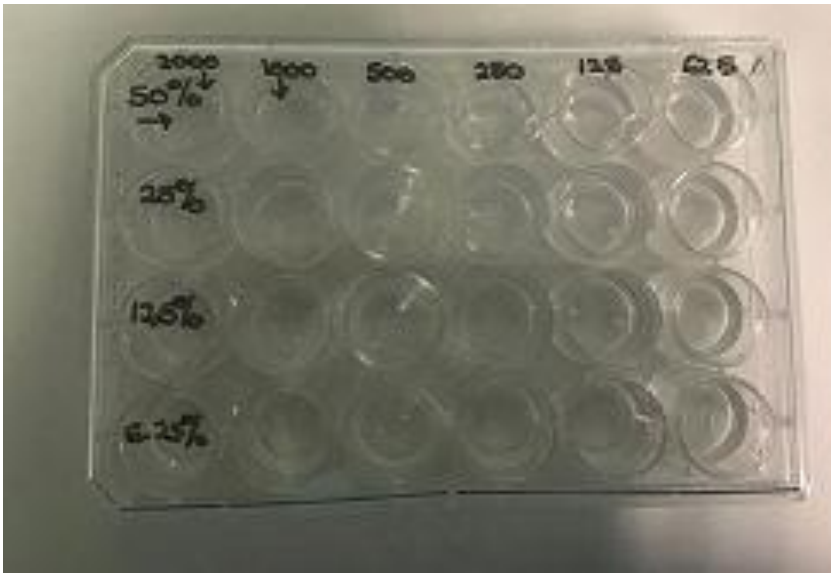

Figure 4: Illustration of a 24 well tissue culture plate with *K. pneumoniae* inocula in dextrose

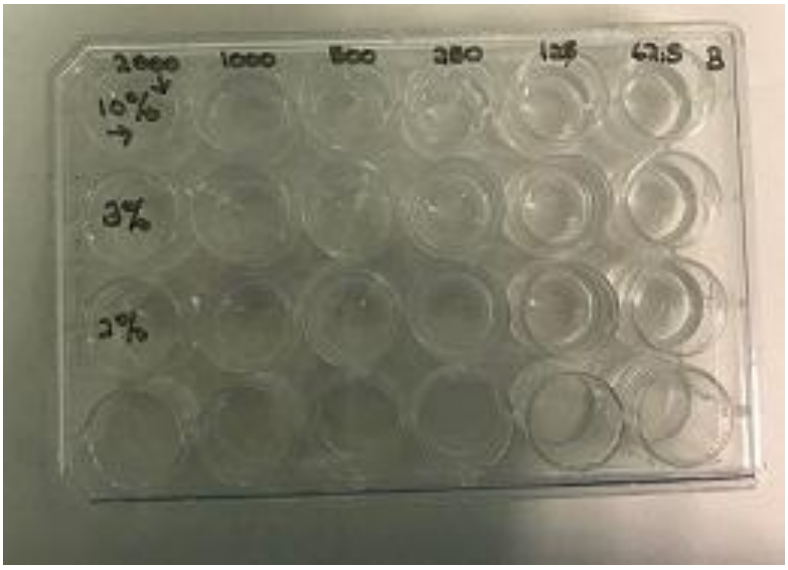

Figure 5: Illustration of a 24 well tissue culture plate with *K. pneumoniae* inocula in dextrose

The *K. pneumoniae* (ATCC 70063) suspension was prepared as described above 4.3.2.1. Two-fold serial dilutions were made resulting in suspensions with six different concentrations of approximately 20000, 10000, 5000, 2500, 1250 and 625 cfu/mL respectively. To the wells of each column of the tissue culture plates A and B, 100 µL of the six *K. pneumoniae* suspensions was added (Figure 4 and 5).

Samples of 100 µL were taken from each well at 0.5 hour and at 1 hour post inoculation. This was followed by hourly sampling for up to eight hours and then after 16 hours. Samples were inoculated onto a MacConkey plate and spread out using disposable loops. All plates incubated overnight, aerobically at 35 ^{o}C. The next day colonies were counted.

CHAPTER FIVE

RESULTS

5.1 Growth curve and turbidity standard curve for *Klebsiella pneumoniae.*

K. pneumoniae ATCC 700603 was incubated aerobically in brain heart infusion broth at 25 ^{o}C and 35 ^{o}C. The number of colony forming units per mL and turbidity at OD_{600} was assessed over a 10 hour period. The data were used to compare growth rates at 25 ^{o}C and 35 ^{o}C and to construct a turbidity standard curve at both temperatures. These standard curves allowed turbidity values to be used instead of more labour intensive colony counts in the experiments to follow.

Figure 6 shows both growth curves at 25 ^{o}C and 35 ^{o}C. At both temperatures, the curve starts with a lag phase of 2 hours at 35 ^{o}C and 3 hours 25 ^{o}C. This was followed by exponential growth at both temperatures resulting in log phases that run in parallel. At 25 ^{o}C, a delay before the exponential phase is observed, whereas at 35 ^{o}C, the exponential phase commences more sharply. Since the aim of this experiment was to establish the correlation between turbidity and bacterial concentration, the incubation was stopped at the end of the exponential phase.

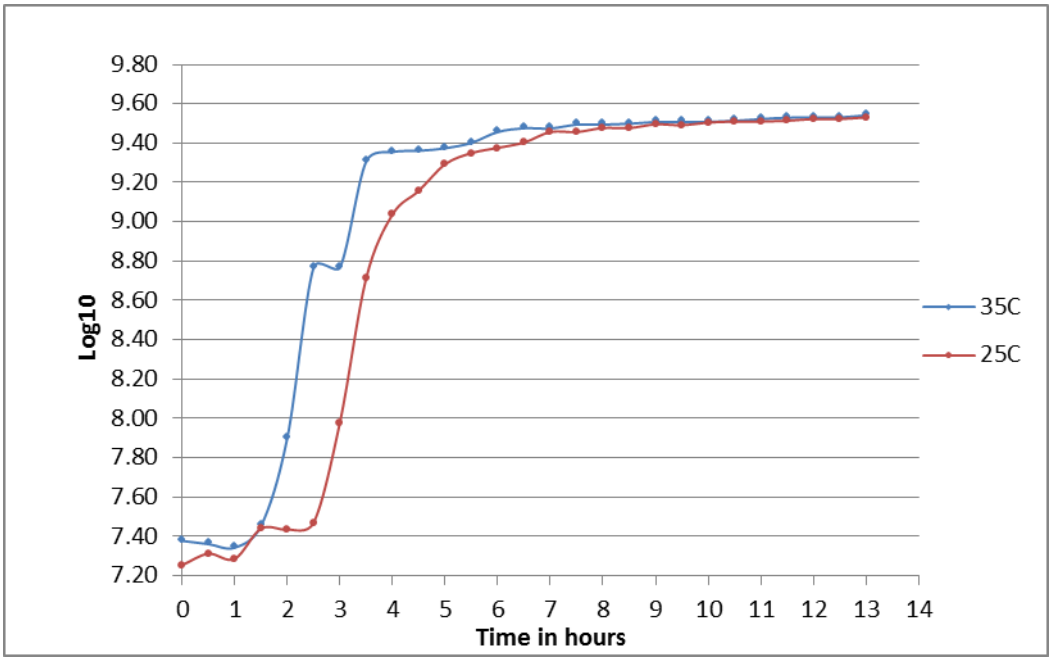

Figure 6: Growth curve *of K. pneumoniae* at 25 ^{o}C and 35 ^{o}C

Figures 7 and 8 shows the relationship between cfu/mL and OD_{600} readings in the log phase of growth at 25 ^{o}C and 35 ^{o}C. The cells in the suspension cause the light to scatter. (63) As the number of cells increase, the scatter of light also increases and with that, the OD reading. Both graphs show the expected linear relationship between the two values.

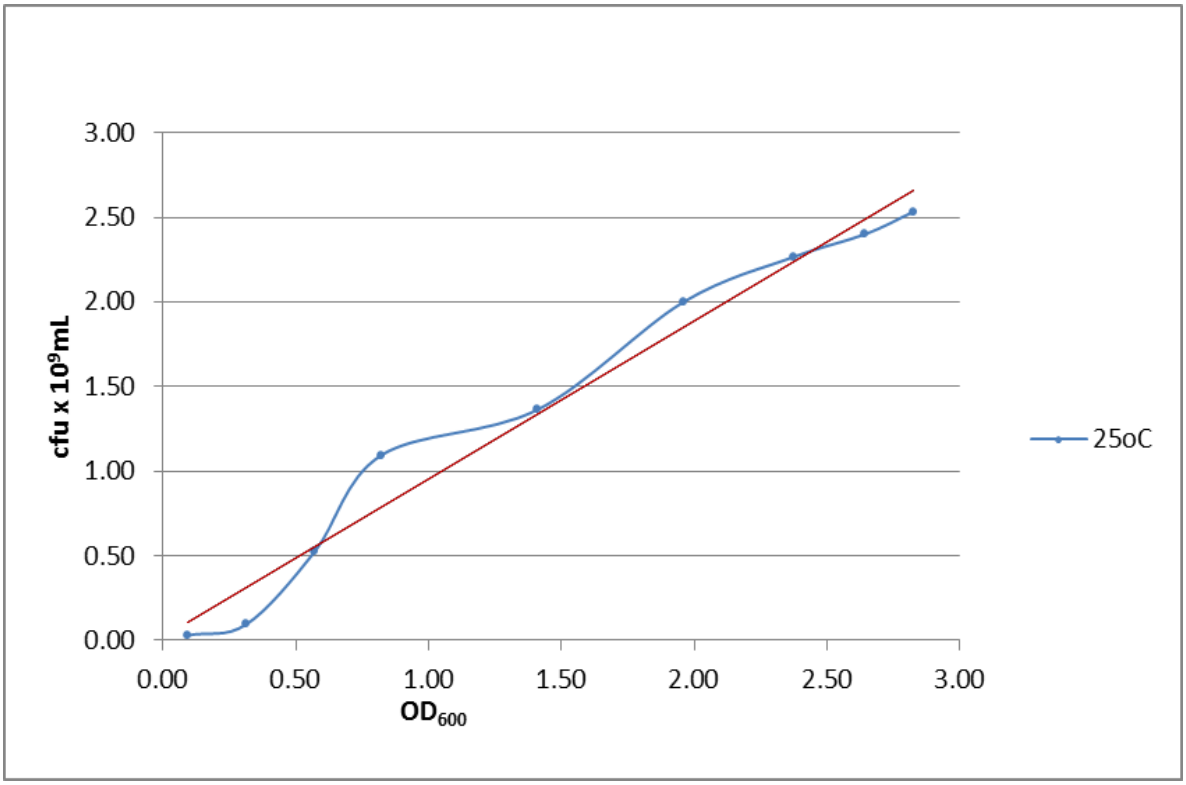

Figure 7: Relationship between optical density at 600 nm and cfu/mL at 25 ^{o}C

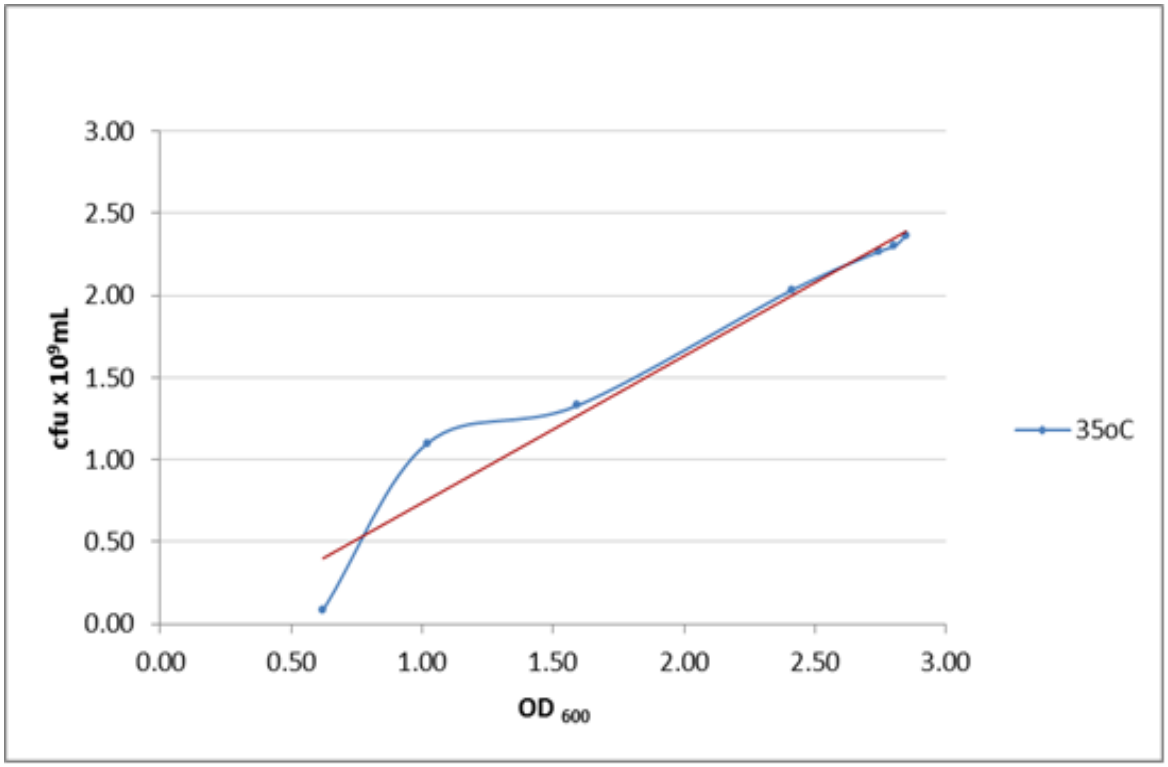

Figure 8: Relationship between optical density at 600 nm and cfu/mL at 35 ^{o}C

An OD of 1.5 was used for all experiments. This corresponds with approximately 1.2 x10^{9} cfu/mL, independent of the incubation temperature. For each experiment this suspension was diluted till the required number of cfu/mL.

5.2 Microbiological challenge test

Table 1 shows the quantitative culture results of the challenge tests. *K. pneumoniae* was isolated up to 1 hour post contamination of the rubber septa of the insulin, heparin, lignocaine**,** Neonatalyte® and saline. In all five, the number of cfu/mL decreased over time, reaching 0 cfu/mL in 1.5 hours post contamination with four of five products. With insulin, growth was only observed after 0.5 hours. With piperacillin-tazobactam, ciprofloxacin and meropenem as well as with gentamicin and 50 % dextrose no growth was observed at any time point

Table 1: Quantitative culture results (cfu/mL) of the challenge tests

	Insulin	**Heparin**	**Lignocaine**	**Neonatalyte®**	**Saline**
Time in (Hr)					
0.5	8	46	22	45	12
1	0	2	12	1	2
1.5	0	0	0	0	0

Results of the challenge tests at all time points are shown in appendix D.

The impression cultures of the rubber septa of the vials and intravenous ports of intravenous fluids showed no growth at 24 hours post experimental contamination.

5.3 K. pneumoniae inoculated in medication fluids

Tables 2 and 3 show the results. Insulin, heparin and lignocaine failed to support the growth of *K. pneumoniae*. When inoculated with 1000 cfu/mL, the bacterial density at 25 ^{o}C decreased by 65 %, 33 % and 20 % respectively whereas at 35 ^{o}C, the cfu/mL decreased with 83 %, 97 % and 81 % respectively. The bactericidal activity of these solutions is presented in tables two and three. In 5000 IU/mL of heparin, the bacterial count declined in the first 30 minutes rapidly to 670 cfu/mL and 33 cfu/mL at 25 ^{o}C and 35 ^{o}C respectively. It took an additional 5.5 hours and 1.5 hours for the remaining 67 % and 7 % of the inoculum to be killed. This rapid decline in the first 30 min. was also seen with insulin and lignocaine but the percentage survival at 30 min at 25 ^{o}C was 35% for insulin and 80 % for lignocaine. At 35 ^{o}C these figures were lower: 17 % and 19 % respectively. At 25 ^{o}C, no growth was detected at 6 hours post inoculation in insulin and at 7 hours post inoculation in lignocaine whereas at 35 ^{o}C, no growth was detected in insulin and lignocaine at 5 hour and 4 hours respectively. Full results are provided in Appendix E.

Table 2: *K. pneumoniae* inocula in insulin, heparin, lignocaine, Neonatalyte® and saline at 25 ^{o}C.

The cfu/mL and the percentage survival at each time point are shown below.

	Insulin		Heparin		Lignocaine		Neonatalyte		Saline	
Time(Hrs)	cfu/mL	% survive	cfu/mL	% survive	cfu/mL	% survive	cfu/mL	% survive	cfu/mL	% survive
0.5	350	35	670	67	800	80	900	90	950	95
1	120	12	500	50	760	76	908	91	900	90
2	90	9	450	45	400	40	903	90	910	91
3	80	8	300	30	233	23	900	90	870	87
4	50	5	100	10	108	11	908	91	900	90
5	30	3	40	4	87	9	900	90	910	91
6	0	0	40	4	3	0.3	899	90	920	92
7	0	0	30	3	0	0	970	97	950	95
8	0	0	0	0	0	0	800	80	900	90
24	0	0	0	0	0	0	2860	286	890	89

Table 3: *K. pneumoniae* inocula in insulin, heparin, lignocaine, Neonatalyte® and saline at 35 ^{o}C

The cfu/mL and the percentage survival at each time point are shown below.

	Insulin		Heparin		Lignocaine		Neonatalyte		Saline	
Time(Hrs)	cfu/mL	% survive	cfu/mL	% survive	cfu/mL	% survive	cfu/mL	% survive	cfu/mL	% survive
0.5	173	17	33	3	190	19	630	63	773	77
1	57	6	3	0.3	153	15	757	76	603	60
2	33	3	0	0	87	9	690	69	533	53
3	7	0.7	0	0	5	0.5	710	71	507	51
4	3	0.3	0	0	0	0	700	70	591	59
5	0	0	0	0	0	0	780	78	520	52
6	0	0	0	0	0	0	720	72	487	49
7	0	0	0	0	0	0	640	64	533	53
8	0	0	0	0	0	0	783	78	503	50
24	0	0	0	0	0	0	4130	413	750	75

In Neonatalyte®, there was an initial decrease of 10 % and 37 % in the cfu/mL at 25 ^{o}C and 35 ^{o}C respectively. At both temperatures, the bacterial density then remained constant up to 8 hours post inoculation. Between 8 and 24 hour an increase in cfu/mL of 286 % and 413 % was detected in at 25 ^{o}C and 35 ^{o}C respectively.

In saline, there was a decrease in the cfu/mL of 5 % and 23 % at 25 ^{o}C and 35 ^{o}C respectively. Thereafter, the cfu/mL remained constant up 24 hours post inoculation. No increase in bacterial density was observed.

The results are shown in Appendix E.

5.4 Dextrose concentration and growth of *K. pneumoniae*

Viability of the K. pneumoniae in dextrose

The behaviour of *K. pneumoniae* was studied in various concentrations of dextrose. This was assessed over a 24 hour period at 25 ^{o}C and 35 ^{o}C. The results with an inoculum of 1000 cfu/mL are shown in tables 4 and 5. Full results are in appendix F.

Bactericidal activity was observed in 50 %, 25 % and 12.5 % dextrose at both incubation temperatures. In 50 % dextrose a reduction in colony count of 39 % was observed after 30 min at 25 ^{o}C and of 57 % at 35 ^{o}C. In 25 % dextrose, there was no change in the bacterial density and in 12.5 % dextrose, a slight decrease in the bacterial density of 5 % at 25 ^{o}C and 6 % at 35 ^{o}C was observed. No growth was observed in 50 %, 25 % and 12.5 % dextrose at 25 ^{o}C in 3 hours, 4 hours and 24 hours post inoculation respectively whereas at 35^{o}C, this was observed in 4 hours and 5 hours in 50 % and 25 % dextrose respectively. No growth was also observed 24 hours post inoculation in 12.5 % dextrose at 35^{o}C as well.

In 10 % dextrose, there was no change in the bacterial density in 30 min. In one hour, there was a decrease in the cfu/mL of 32 % and 1 % at 25 ^{o}C and 35 ^{o}C respectively. Thereafter, a gradual decrease in the bacterial density was observed. At 24 hours post inoculation, a decrease of 57 % and 50 % was observed at 25 ^{o}C and 35 ^{o}C respectively.

In the lower concentration of dextrose of 6 %, 3 % and 2 %, there was an initial decrease in the colony counts at both temperatures up to 8 hours post inoculation. At 25 ^{o}C, the decrease in the colony count ranged from 1-13 % and at 35 ^{o}C, this was 1-22 %. After 24 hours post inoculation at 25 ^{o}C, in the 6 %, 3 % and 2 % the colony counts increased by 12 %, 24 % and 33 % respectively. At 35^{o}C, slightly higher increases were observed with 20 % in the 6 % dextrose and 34 % in the 3 % dextrose at 24 hours post inoculation. The 2 % dextrose, however, showed a 1 % increase in the bacterial density by 6 hours post inoculation and by 24 hours post inoculation 50 % increase in cfu/mL.

Table 4: *K. pneumoniae* inocula in dextrose at 25 ^{o}C

The number of cfu/mL and percentage survival is shown in the table below.

	50 %		25 %		12.50 %		10 %		6 %		3 %		2 %	
Time(Hr)	cfu/mL	%	cfu/mL	%	cfu/mL	%	cfu/mL	%	cfu/mL	%	cfu/mL	%	cfu/mL	%
0.5	610	61	1000	100	950	95	1000	100	950	95	980	98	990	99
1	550	55	610	61	810	81	680	68	980	98	980	98	910	91
2	50	5	520	52	540	54	660	66	870	87	970	97	940	94
3	0	0	40	4	520	52	640	64	880	88	980	98	980	98
4	0	0	0	0	560	56	640	64	890	89	990	99	980	98
5	0	0	0	0	560	56	590	59	910	91	910	91	960	96
6	0	0	0	0	430	43	570	57	940	94	940	94	940	94
7	0	0	0	0	480	48	590	59	980	98	980	98	980	98
8	0	0	0	0	300	30	450	45	980	98	980	98	990	99
24	0	0	0	0	0	0	430	43	1120	112	1240	124	1330	133

Table 5: *K. pneumoniae* inocula in dextrose at 35 ^{o}C

The number of colonies and percentage survival is shown in the table below

	50 %		25 %		12.50 %		10 %		6 %		3 %		2 %	
Time(Hr)	cfu/mL	%	cfu/mL	%	cfu/mL	%	cfu/mL	%	cfu/mL	%	cfu/mL	%	cfu/mL	%
0.5	430	43	1000	100	940	94	1000	100	880	88	980	98	910	91
1	230	23	430	43	750	75	990	99	800	80	940	94	980	98
2	70	7	230	23	470	47	960	96	780	78	950	95	980	98
3	10	1	70	7	410	41	930	93	900	90	910	91	970	97
4	0	0	10	1	440	44	760	76	900	90	930	93	960	96
5	0	0	0	0	480	48	680	68	940	94	990	99	980	98
6	0	0	0	0	380	38	640	64	930	93	980	98	1010	101
7	0	0	0	0	320	32	620	62	930	93	970	97	1200	120
8	0	0	0	0	360	36	600	60	940	94	980	98	1180	118
24	0	0	0	0	0	0	500	50	1200	120	1340	134	1500	150

CHAPTER SIX

DISCUSSION

Patient safety forms an essential part of ensuring quality healthcare and is the foundation of a strong system. (64) The World Health Organisation(WHO) states that, "Unsafe medication practices and medication errors are a leading cause of injury and avoidable harm in health care systems across the world." (65) In medical practice, medications are often administered via injections. (66) Micro-organisms can easily be introduced into the blood stream or tissues in this way. A safe injection does not harm the recipient, does not expose the provider to any avoidable risks and does not result in waste that is dangerous for the community or the environment. (66)

Multiple-dosing of single use medication is a dangerous practice. Medications meant for single dosing do not have a preservative. Depending on the constituents of the medication fluids and concentration, these medication fluids may allow bacterial growth or bacterial persistence such as Neonatalyte® and isotonic solution. (67)

Experiments that determine behavior of organism in medication fluids require that these organisms are in the log phase of growth at the time of inoculation. The traditional colony counting method to determine inoculum sizes takes two to three days. In this method ten-fold serial dilution of the culture are plated, incubated and counted. By the time the cfu/mL is determined the culture is no longer in the log phase. (68) Figures 7 and 8 shows a linear relationship of *Klebsiella pneumoniae,* in the log phase, between OD_{600} and cfu/mL. Zhang *et al* had similar findings. (68) Authors used three strains of Gram-negative bacteria (*Escherichia coli, K. pneumoniae,* and *Pseudomonas aeruginosa*) and three strains of Gram-positive bacteria (*Staphylococcus aureus, Enterococcus faecium,* and *Bacillus subtilis*) to construct growth curves. (68)

Table 1 show the results of the microbiological challenge test. It is possible that the organisms isolated were either from the rubber septa and medication bag ports or the medication fluid. The septa of medication vial are designed to have a self-sealing ability and in the case of multiple-dose vials, be able to withstand multiple withdrawals. (27) It is most probable that the organisms were from the rubber septa.

When determining the growth kinetics of *K. pneumoniae* in fluids containing preservatives; (insulin, heparin and lignocaine) the fluids showed bactericidal activity at both incubation temperatures. The 3 log reduction meets the standards for antimicrobial effectiveness. (69, 70) The bactericidal activity demonstrated at 25 ^{o}C indicates that these vials can be safely stored at this temperature. Bactericidal

activity of preservative containing medication was also demonstrated by Highsmith *et al* (47). However, in this study an extended persistence of *K. pneumoniae* of up to 24 hours post inoculation was found in lignocaine. Longfield *et al* (48) demonstrated a mean bacterial killing time of 5.3 hours in insulin, 5.4 hours in heparin and 15.5 hours in lidocaine. This study showed an even faster time to sterility in lignocaine. When stored at the recommended storage temperatures, these medications have not shown contamination. A study has shown that storage of multiple-dose vials at 4 ^{o}C adversely affects the bacterial activity of the preservative. (12) Because of this finding, multiple-dose vials stored in the refrigerator have been hypothesized to be the source of outbreaks. (12) The current KwaZulu-Natal (KZN) policy recommends that multiple-dose vials be stored in a refrigerator at 4 ^{o}C. (10) This policy requires revision to specify storage at room temperature or as stipulated by the manufacturer.

Heparin is a mucopolysaccharide composed of partially sulfated units of glucuronic acid and aminodeoxyglucose. (71) Rosett *et al* showed the ability of heparin to inhibit the growth of microorganisms. (72) It was hypothesized that heparin binds cations essential for bacterial growth. (72)

Whilst the antibacterial activity of heparin has been demonstrated, contamination by other micro-organisms was not excluded. Saigul *et al* (73) reported that a young girl was infected with *Plasmodium falciparum* after she received heparin from a multiple-dose vial. The heparin solution was contaminated as a result of re-use of syringes. The syringe was contaminated with blood from a malaria patient. The infected blood was then introduced into the vial and subsequently into the patient. (73) The preservative used in heparin is not effective against protozoa. Proteins are able to inactivate preservatives and provide bacteria with nutrition necessary for growth. A combination of these factors could have resulted in pathogenic reservoirs in medication vials.

Fluids meant for multiple-dosing contain preservatives. Provided these vials were used and stored in accordance with the manufacturer's instructions, contamination rates remain low. (74) The one vial, one patient approach is the safest. In low resource settings, where this is not feasible, clear guidelines must be provided for safe use of medication fluids with consideration on a product to product basis.

Administration of intravenous fluid can provide an opportunity for introduction of bacteria into the blood stream. Bacteria can enter the fluid during preparation of the medication, administration of the medication and subsequent handling of the bag and ports. These medications do not contain preservatives and thus present as a major risk factor for blood stream infections. The ability of *K. pneumoniae* to persist in Neonatalyte® and in saline must be considered when developing guidelines. Due to the volume of the bags, these fluids are often used for multiple-dosing in paediatric patients. (26) The current KwaZulu-Natal policy allows multiple-dosing of preservative free, single use medications, provided withdrawals

are made one after the other. (10) Outbreaks have been reported with heparin/saline flushes and multi-dosing of Neonatalyte® and saline. (31, 34, 39, 75) Bacteria inadvertently introduced into these bags can cause healthcare associated infection and outbreaks. It is critical that this policy be revised into single patient, single use. Health care professionals must be made aware of the risks.

Fifty percent, 25 %, 12.5 % and 10 % dextrose solutions are hypertonic. The hypertonic nature of the solution can draw fluid out of the bacterial cell, leading to dehydration of bacteria and eventual death. The 12.5 % solution of dextrose allowed persistence of *K. pneumoniae* up to 8 hours, whilst 10 % allowed persistence for up to 24 hours.

The lower concentrations are isotonic in nature. The cellular integrity is thus not affected by the solution. Also, utilisation of carbon is said to be optimal in a glucose/dextrose limited environment. (76) These two factors could account for the increase in cfu/mL. The log increase at 25^{o}C in 6 % dextrose was 0.04, in 3 % 0.09 and in 2 % was 0.12. Lack of bacterial growth is defined as no more than a 0.5-$\log_{10}$ increase. (70, 77) Therefore, the increases in cfu/mL in these low dextrose concentrations are not regarded bacterial growth. Whilst this solution allows for metabolism, it does not promote rapid proliferation. Survival of *K. pneumoniae* is inversely related to the concentration of dextrose.

Dextrose is used for management of hypoglycaemia and in total parenteral nutrition (TPN). In paediatrics, dextrose is used at concentrations of 2.5 to 10 %. (78) At both concentrations, bacteria persist. Results similar to ours were reported by Hugbo *et al*. (67) In that study, *K. pneumoniae* decreased with increasing dextrose concentrations. *C. albicans* was also included in the panel of test organisms and the cell count of that organism remained constant at all dextrose concentrations. The conclusion reached was that with bacteria, the extent of inhibition of growth was dependent on the dextrose concentration, incubating conditions and pH of dextrose solutions whereas yeasts are not affected by dextrose concentration. (67)

This study tested only *K. pneumoniae* ATCC 700603. This is a well characterised extended spectrum beta lactamase producer. This organism was chosen because in KZN, this is the sole cause of outbreaks due to the use of medication. In future, the study should be extended with other micro-organisms as well as with surveillance studies of in-use vials to assess contamination rates in the health care setting.

Whilst proliferation has not been demonstrated, persistence of the test bacteria in isotonic saline, Neonatalyte® and lower concentrations of dextrose has been shown. This poses serious risk to patient care if the bacteria inadvertently enter the fluid due to infection prevention breeches.

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APPENDICES

APPENDIX A

UNIVERSITY OF
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11 May 2016

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Dear Prof Moodley

PROTOCOL: Provincial Surveillance for Hospital Acquired Infections and Outbreak Investigation. REF: BE222/13.

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved: 06 August 2016
Expiration of Ethical Approval: 05 August 2017

I wish to advise you that your application for Recertification received on 06 May 2016 for the above protocol has been noted and approved by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The approval will be ratified by a full Committee at a meeting to be held on **14 June 2016.**

Yours sincerely

Mrs A Marimuthu
Senior Administrator Biomedical Research Ethics Committee

APPENDIX B

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07 June 2017

Ms R Misra
Infection prevention and control/Medical Microbiology
misra@ukzn.ac.za
moodleyp@ukzn.ac.za (PI of Study)

Dear Ms Misra

PROTOCOL: Provincial Surveillance for Hospital Acquired Infections and Outbreak Investigation. REF: BE222/13. (Class Approval)

NEW TITLE: ***Proliferation of Klebsiella pneumoniae in medication containing vials for multiple dose medication and intravenous fluids. (Sub-study of class approval BE222/13)***

Your correspondence received on 17 May 2017 submitting an application to change your title to the above has been noted and approved by a subcommittee of the Biomedical Research Ethics Committee.

The approval will be ratified by a full Committee at a meeting to be held on 11 July 2017.

Yours sincerely

Ms A Marimuthu
Senior Administrator: Biomedical Research Ethics

cc: Prof AW Sturm (supervisor)

APPENDIX C

Media Preparation

1. Maconkey agar (CM007) Oxoid, Basingstoke, Hampshire, England)
    a. Constituents
        i. MacConkey agar powder 52 g
        ii. Distilled water 1 L
    b. Method
        i. 52 g of Maconkey agar powder was weighed(Radwag Model: WTB 2000, Radom, Poland)and transferred into a glass flask
        ii. 1L of distilled water was measured and added to the flask
        iii. The flask was swirled until the powder was well dissolved
        iv. The opening of the flask was then covered with an acceptor bag and taped with autoclave tape
        v. The media was then autoclaved at 121 ^{o}C (Sterilizer Technologies SD 158) for 15 min
        vi. The flask was then placed in a water bath at 55^{o}C for approximately one hour or until cool enough to touch
        vii. Using a media dispenser (Jencon Perimatic GP Model: GP1, Bridgeville, Unites States of America)and aseptic procedure, 20 mL of the media was dispensed into 90 mm petri dishes(Concorde Plastics (Pty) Ltd, Longdale, Johannesburg, South Africa)
2. 5 % Horse Blood agar
    a. Constituents
        i. Columbia Blood agar base powder 39 g
        ii. Distilled water 1 L
        iii. Horse Blood 50mL
    b. Method
        i. 30 g of Columbia Blood agar base (CM0331)(Oxoid, Basingstoke, Hampshire, England) was weighed and transferred into a glass flask
        ii. 1L of distilled water was measured and added to the flask
        iii. The flask was swirled until the powder was well dissolved
        iv. The opening of the flask was then covered with an acceptor bag and taped with autoclave tape
        v. The media was then autoclaved at 121 ^{o}C for 15 min

vi. The flask was then placed in a water bath at 55 ^{o}C for approximately one hour or until cool enough to touch

vii. Using a media dispenser and aseptic procedure, 20 mL of the media was dispensed into 90 mm petri dishes

3. Storage media
    a. Constituents
        i. Brain Heart Infusion (BHI)broth 12 g
        ii. Distilled water 400 mL
        iii. Glycerol 100 mL
        iv. Glass beads
    b. Method
        i. 12 gr of BHI (Oxoid, Basingstoke, Hampshire, England) powder was weighed and transferred into a glass screw cap bottle(Pyrex)
        ii. 400 mL of distilled water was measured and added to the glass bottle
        iii. The bottle was swirled until the powder was well dissolved
        iv. 100 mL of glycerol was then added to the bottle
        v. The bottle was loosely closed and taped with autoclave tape from the cap to the bottle
        vi. The media was then autoclaved at 121 ^{o}C for 15 min
        vii. The media was then cooled
        viii. The cryovials were then aseptically filled halfway with the glass beads
        ix. 1000 mL of the storage media was dispensed into the cryovials with beads.
        x. Vials were then stored between 2 ^{o}-8 ^{o}C

4. Phosphate buffered Saline(DulbeccoA), (Oxoid, Basingstoke, Hampshire, England)
    a. Constituents
        i. Phosphate Buffered Saline tablets
        ii. 500 mL distilled water
    b. Method

        i. 500 mL of distilled water was dispensed in screw cap glass bottle
        ii. 5 tablets of phosphate buffered saline(PBS) was then added
        iii. The bottle was swirled until well dissolved

iv. The bottle was then loosely capped and an autoclave tape was placed from the cap to the bottle
v. The bottle was sterilised at 115 ^{o}C for 10 min
vi. Once cooled, the 5mL PBS was aseptically dispensed into white screw cap tubes (Whitehead Scientific, Cape Town)

5. Brain Heart Infusion(BHI)(CM135)-(Oxoid, Basingstoke, Hampshire, England)
    a. Constituents
        i. 37g of BHI Powder
        ii. 1L of distilled water
    b. Method
        i. 1000 mL of distilled water was dispensed in screw cap glass bottle
        ii. 37g of BHI powder was added to the glass bottle
        iii. The bottle was swirled until well dissolved
        iv. The bottle was then loosely capped and an autoclave tape was placed from the cap to the bottle
        v. The bottle was sterilised at 121 ^{o}C for 15 min

APPENDIX D

Table 6: This table illustrates the number of colonies isolated at each time point in cfu/mL. The time point is indicated in hours-post spiking.

	Tazobax	**Ciprocina**	**Meronem**	**Insulin**	**Heparin**	**Dextrose 50%**	**Gentamycin**	**Neonalyte®**	**NaCl**	**Lignocaine**
Time: (Hr)										
0.5	0	0	0	8	46	0	0	45	12	22
1	0	0	0	0	2	0	0	1	2	12
1.5	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0
2.5	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0
3.5	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0
4.5	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0
5.5	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0
6.5	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0
7.5	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0

APPENDIX E -Table 7: *K. pneumoniae* inocula in insulin at 25 ^{o}C and 35 ^{o}C.

The cfu/mL and the percentage survival at each time point are shown below.

cfu/mL	**1000**		**500**		**250**		**125**		**62**		**31**	
Time (Hrs)												
	25^{o}C	35^{o}C	25^{o}C	35^{o}C	25^{o}C	35^{o}C	25^{o}C	35^{o}C	25^{o}C	35^{o}C	25^{o}C	35^{o}C
0.5	350	173	300	150	80	30	20	10	10	3	0	0
1	120	57	276	124	50	10	1	0	4	0	0	0
2	90	33	100	50	4	0	0	0	0	0	0	0
3	80	7	90	45	0	0	0	0	0	0	0	0
4	50	3	100	33	0	0	0	0	0	0	0	0
5	30	0	96	21	0	0	0	0	0	0	0	0
6	0	0	9	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0

Table 8: *K. pneumoniae* inocula in heparin at 25 ^{o}C and 35 ^{o}C .

The cfu/mL and the percentage survival at each time point are shown below.

cfu/mL	**1000**		**500**		**250**		**125**		**62**		**31**	
Time:Hrs												
	25°C	35°C	25°C	35°C	25°C	35°C	25°C	35°C	25°C	35°C	25°C	35°C
0.5	670	33	403	201	230	115	100	50	23	10	12	6
1	500	3	190	90	90	45	0	2	0	0	1	0
2	450	0	45	21	8	4	0	0	0	0	0	0
3	300	0	10	5	0	0	0	0	0	0	0	0
4	100	0	4	2	0	0	0	0	0	0	0	0
5	40	0	0	0	0	0	0	0	0	0	0	0
6	40	0	0	0	0	0	0	0	0	0	0	0
7	30	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0

Table 9: *K. pneumoniae* inocula in lignocaine at 25 ^{o}C and 35 ^{o}C

The cfu/mL and the percentage survival at each time point are shown below.

cfu/mL	**1000**		**500**		**250**		**125**		**62**		**31**	
Time:Hrs												
	25^{o}C	35^{o}C	25^{o}C	35^{o}C	25^{o}C	35^{o}C	25^{o}C	35^{o}C	25^{o}C	35^{o}C	25^{o}C	35^{o}C
0.5	800	190	300	145	200	100	100	30	8	0	1	0
1	760	153	190	110	180	87	0	2	0	0	1	0
2	400	87	90	40	130	65	0	0	0	0	0	0
3	233	5	8	4	90	45	0	0	0	0	0	0
4	108	0	4	1	2	0	0	0	0	0	0	0
5	87	0	0	0	1	0	0	0	0	0	0	0
6	3	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0

Table 10: *K. pneumoniae* inocula in Neonatalyte® at 25 ^{o}C and 35 ^{o}C

The cfu/mL and the percentage survival at each time point are shown below

cfu/mL	**1000**		**500**		**250**		**125**		**62**		**31**	
Time:Hrs												
	25^{o}C	35^{o}C	25^{o}C	35^{o}C	25^{o}C	35^{o}C	25^{o}C	35^{o}C	25^{o}C	35^{o}C	25^{o}C	35^{o}C
0.5	900	630	489	450	209	230	100	128	8	6	1	9
1	908	757	450	430	180	201	6	110	0	2	1	0
2	903	690	350	420	130	190	0	67	0	0	0	0
3	900	710	490	439	90	87	0	9	0	0	0	0
4	908	700	510	398	2	56	0	0	0	0	0	0
5	900	780	530	386	1	0	0	0	0	0	0	0
6	899	720	348	365	0	0	0	0	0	0	0	0
7	970	640	450	378	0	0	0	0	0	0	0	0
8	800	783	540	459	0	0	0	0	0	0	0	0
24	2860	4130	509	430	0	0	0	0	0	0	0	0

Table 11: *K. pneumoniae* inocula in saline at 25 ^{o}C and 35 ^{o}C

The cfu/mL and the percentage survival at each time point are shown below.

cfu/mL	**1000**		**500**		**250**		**125**		**62**		**31**	
Time:Hrs	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**
0.5	950	773	475	498	200	256	100	135	30	56	1	9
1	900	603	400	458	200	250	90	110	45	34	1	0
2	910	533	425	415	190	189	90	109	2	2	0	0
3	870	507	375	425	180	176	80	98	1	0	0	0
4	900	591	300	309	170	165	0	45	0	0	0	0
5	910	520	275	320	165	150	0	4	0	0	0	0
6	920	487	250	289	150	245	0	0	0	0	0	0
7	950	533	225	289	160	230	0	0	0	0	0	0
8	900	503	220	296	170	235	0	0	0	0	0	0
24	890	750	200	398	160	230	0	0	0	0	0	0

APPENDIX F

Table 12: *K. pneumoniae* inocula in 50 % dextrose at 25 ^{o}C and 35 ^{o}C.

The cfu/mL and the percentage survival at each time point are shown below

cfu/mL	**1000**		**500**		**250**		**125**		**62**		**31**	
Time:Hrs	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**
0.5	610	430	113	203	93	87	47	17	17	10	0	0
1	550	230	57	87	7	13	0	0	0	0	0	0
2	50	70	3	3	0	0	0	0	0	0	0	0
3	0	10	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0

Table 13: *K. pneumoniae* inocula of 25 % dextrose in at 25 ^{o}C and 35 ^{o}C

The cfu/mL and the percentage survival at each time point are shown below.

cfu/mL	**1000**		**500**		**250**		**125**		**62**		**31**	
Time:Hrs	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**
0.5	1000	1000	310	270	110	30	10	3	3	3	0	0
1	610	430	165	20	0	0	0	0	0	0	0	0
2	520	230	6	0	0	0	0	0	0	0	0	0
3	40	70	0	0	0	0	0	0	0	0	0	0
4	0	10	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0

Table 14: *K. pneumoniae* inocula in12.5 % dextrose at 25 ^{o}C and 35 ^{o}C.

The cfu/mL and the percentage survival at each time point are shown below

cfu/mL	**1000**		**500**		**250**		**125**		**62**		**31**	
Time:Hrs	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**
0.5	950	940	460	410	210	230	0	0	0	0	0	0
1	810	750	300	400	200	100	0	0	0	0	0	0
2	540	470	340	390	173	100	0	0	0	0	0	0
3	520	410	330	370	90	10	0	0	0	0	0	0
4	560	440	290	360	43	13	0	0	0	0	0	0
5	560	480	340	360	6	3	0	0	0	0	0	0
6	430	380	280	330	0	0	0	0	0	0	0	0
7	480	320	330	313	0	0	0	0	0	0	0	0
8	300	360	240	160	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0

Table 15: *K. pneumoniae* inoculum in 10 % dextrose at 25 ^{o}C and 35 ^{o}C

The cfu/mL and the percentage survival at each time point are shown below.

cfu/mL	**1000**		**500**		**250**		**125**		**62**		**31**	
Time:Hrs	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**
0.5	1000	1000	360	400	190	183	100	90	3	0	6	3
1	680	990	340	370	150	116	30	60	0	0	0	0
2	660	960	320	320	130	120	30	10	0	0	0	0
3	640	930	300	320	120	90	6	3	0	0	0	0
4	640	760	270	300	110	70	3	3	0	0	0	0
5	590	680	260	260	100	66	3	10	0	0	0	0
6	570	640	260	250	90	60	0	3	0	0	0	0
7	590	620	200	200	100	50	3	10	0	0	0	0
8	450	600	150	190	80	40	3	0	0	0	0	0
24	430	500	180	90	60	20	0	0	0	0	0	0

Table 16: *K. pneumoniae* inocula in 6 % dextrose at 25 ^{o}C and 35 ^{o}C

The cfu/mL and the percentage survival at each time point are shown below.

cfu/mL	**1000**		**500**		**250**		**125**		**62**		**31**	
Time:Hrs	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**
0.5	950	880	460	470	210	176	126	120	86	0	0	3
1	980	800	380	450	200	146	123	100	10	0	0	0
2	870	780	410	460	180	120	110	120	0	0	0	0
3	880	900	446	490	230	160	130	140	0	0	0	0
4	890	900	403	500	240	200	130	120	0	0	0	0
5	910	940	490	530	230	210	130	130	0	0	0	0
6	940	930	480	530	250	200	130	110	0	0	0	0
7	980	930	600	650	280	230	120	120	0	0	0	0
8	980	940	610	690	310	240	130	160	0	0	0	0
24	1120	1200	636	820	360	223	140	170	0	0	0	0

Table 17: *K. pneumoniae* inocula in 3 % dextrose at 25 ^{o}C and 35 ^{o}C

The cfu/mL and the percentage survival at each time point are shown below.

cfu/mL	**1000**		**500**		**250**		**125**		**62**		**31**	
Time:Hrs	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**
0.5	980	980	360	390	210	180	120	120	70	40	0	0
1	980	940	380	436	200	190	100	120	10	20	0	0
2	970	950	410	430	190	210	150	110	3	3	0	0
3	980	910	440	390	290	240	110	100	3	0	0	0
4	990	930	400	390	240	250	110	100	0	0	0	0
5	910	990	490	410	230	280	90	116	0	0	0	0
6	940	980	480	450	250	270	130	110	0	0	0	0
7	980	970	450	446	280	280	130	120	0	0	0	0
8	980	980	410	500	300	310	126	130	0	0	0	0
24	1240	1340	880	940	340	410	130	166	0	0	0	0

Table 18: *K. pneumoniae* inoculum in 2 % dextrose in at 25 ^{o}C and 35 ^{o}C

The cfu/mL and the percentage survival at each time point are shown below.

cfu/mL	**1000**		**500**		**250**		**125**		**62**		**31**	
Time:Hrs	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**	**25^{o}C**	**35^{o}C**
0.5	990	910	490	510	190	180	130	130	70	66	0	0
1	910	980	486	546	290	153	126	90	36	20	0	0
2	940	980	410	560	240	240	123	160	33	16	0	0
3	980	970	400	580	230	103	110	130	23	10	0	0
4	980	960	410	490	250	100	119	130	16	10	0	0
5	960	980	500	600	180	100	123	140	3	6	0	0
6	940	1010	496	680	150	180	126	150	0	0	0	0
7	980	1200	460	690	110	186	120	160	0	0	0	0
8	990	1180	490	600	130	180	120	150	0	0	0	0
24	1330	1500	900	900	290	290	126	170	0	0	0	0