Chapter 8

Application of ECA solutions to control nosocomial infections in a Neonatal Intensive Care Unit

8.1 Abstract

In response to frequent outbreaks of *Klebsiella spp* infection in the Neonatal Intensive Care Unit (NICU) in a large referral hospital in Botswana, it was proposed that the existing infection control strategy be augmented with the inclusion of an Electro-Chemically Activated (ECA) oxidant water solution - Actsol®[1], for the disinfection of equipment and contact surfaces within the facility.

The study was carried out primarily in the Neonatal and Postnatal wards and was later extended to include both the male and female surgical wards. The results confirm that disinfection with the Actsol® solution significantly reduced the incidence of all microorganisms on designated surfaces. Overall, a 57 fold reduction in total viable bacterial count was recorded on all surfaces cleaned with Actsol® solution, while surfaces cleaned according to existing procedures with conventional chemicals only displayed a 7 fold reduction.

The incidence of new *Klebseilla spp*. infections in the NICU was eliminated within one week of the Actsol® intervention and the sources of previously persistent *Klebsiella spp* contamination was eliminated. It appears that the continuous availability of the Actsol® solution in this confined environment was effective in controlling pathogen transmission.

The study confirms that the use of Actsol® can assist in the control of *Klebsiella spp.* outbreaks in neonatal wards, and could be used to enhance the overall hygiene conditions within other hospital wards.

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[1] Actsol® is the registered trade mark of Radical Waters

8.2 Introduction

Nosocomial or Hospital Acquired Infections (HAI) are one of the leading causes of morbidity and mortality in Neonatal Intensive Care Units (Borghesi and Stronati, 2008). Outside of the obvious requirement for optimal sanitization of medical devices and equipment used in the treatment of hospitalized patients, effective cleaning and disinfection of inanimate contact surfaces is likewise critical for the prevention and control of nosocomial infections within a health care environment. This requirement is especially relevant for hospitalised individuals in the high risk categories i.e. neonates and the elderly, but it also has significance where immunocompromised or patients with heightened susceptibility to opportunistic pathogens require protracted hospitalization.

In developed countries, the control of nosocomial infections is largely driven by political, legal and public awareness, while in developing countries, the prevalence of HIV, malaria, tuberculosis and hepatitis is seen as the primary threat to public health and most HAI's are accorded only a limited significance (Hambraeus, 2006). Correspondingly, it was found that the predominant organisms responsible for nosocomial infections in technically advanced countries were gram positive cocci, while gram negative bacilli were the major cause of HAI's in developing countries (Srivastava and Shetty, 2007).

While the multifactorial origins of nosocomial infections have been extensively assessed, most outbreaks can be traced back to either the lack of effective infection control strategies or their inconsistent implementation (Schabrun and Chipchase, 2006; Srivastava and Shetty, 2007). Coupled to this finding is the assertion that the pursuit of cost containment is contributing to the increasing prevalence of inadequately sanitized surfaces in health care facilities (Griffith *et al*., 2000).

While many disinfecting products are available to the health care market, long term use of many compounds including gluteraldehyde, iodophores and phenols have developed application limitations in terms of carcinogenicity, sensitivity and resistance respectively.

Electrolyzed oxidizing water (EOW), Electrolysed strong acid aqueous solution (ESAAS), Electrochemically activated water (ECA) or superoxidised water (SO), has been reported to be broadly antimicrobicidal with proven bactericidal (including MDR strains), sporicidal, fungicidal, virucidal and cysticical attributes (Venczel *et al*., 1997; Shetty *et al*., 1999; Loshan, 2001; Landa-Solis *et al*., 2005).

There has been a recent increase in the number of reports that describe the benefits of the use of ElectroChemically Activated (ECA) solutions for equipment cleaning and disinfection in the health care arena. In dentistry, the technology has been assessed for the disinfection of dental unit water lines, hand sets and endodontic equipment (Marais and Brözel, 1999; Martin and Gallagher, 2005; Wu *et al*., 2008), and its surface use has also been extended to include invasive disinfection interventions involving root canals (Marais, 2000; Solovyeva and Dummer, 2000). In medicine, the disinfecting ability of the ECA solutions has been reported as being a safe and effective substitute for gluteraldehyde in the cleaning and sanitation of endoscopes and similar frequent re-use equipment in health care facilities (Panicheva, 1999; Selkon *et al*., 1999; Shetty *et al*., 1999; Middleton *et al*., 2000; Thanthsa, 2002; Landa-Solis *et al*., 2005). In addition, it has also been used for more invasive medical treatments and a variety of significant health benefits have been ascribed to its distinctive attributes (Hayashi *et al*., 1997; Nakae and Inaba, 2000; Hanaoka, 2001; Landa-Solis *et al*., 2005).

While there are a number of anecdotal reports on the use of the ECA solutions for environmental decontamination of health care facilities (Devyatov *et al*., 1999; Kruglov and Leonov, 1999; Myazitov and Maximov, 1999; Vorobjeva *et al*., 2004), most reports describe specific ECA interventions under simulated conditions where nosocomial or iatrogenic disease conditions might eventuate (Clark *et al.*,2006).

The oxidant ECA solution has been described as being non-irritating and non-sensitising, free of specific fume extraction or protective clothing requirements, and where disposal and spillage can be managed without special precautions (Marais, 2000; Landa-Solis *et al*., 2005). A further advantage is that the solution can be produced on site, as and when required, thus obviating the traditional problems

associated with logistics, storage, handling and shelf life of packaged chemical products (Middleton *et al*., 2000).

8.3 Objectives of the study

The objective of the study was to establish the antimicrobial efficacy of the Actsol® solution within a high risk medical environment, and to assess the capability of the solution to assist in the control of nosocomial infections due to *Klebsiella spp.* in a neonatal ward. A further objective of the study was to qualify the source and to quantify the prevalence of pathogenic organisms capable of causing nosocomial infection from a variety of surfaces in the NICU and the Postnatal Wards (PNW).

Coupled to this was an investigation to quantify the impact that the introduction of the Actsol® ECA solution as a surface cleaning agent would have on the prevalence of pathogenic surface contaminants, and, finally to compare the relative disinfecting efficacy of the Actsol® solution against the conventional sanitizing procedures and products.

8.4 Materials and Methods

The study was initiated in response to regular disease outbreaks due to *Klebsiella spp*. infections in infants admitted to the NICU. Despite dedicated remedial interventions by the resident infection control authority, *Klebsiella spp*. associated nosocomial infections in the NICU persisted, and an emergency intervention with an initial bulk supply of Actsol® was followed up with the on-site installation of continuously piped supply of Actsol® solution to the NICU sluice room. The ECA device was supplied and maintained by Radical Waters, Kyalami, South Africa.

All equipment including disassembled incubators which were routinely washed in the sluice room were terminally rinsed with Actsol®, and the solution was extensively used for the general surface and floor decontamination in the NICU. In addition, all wash hand basins within the NICU were routinely disinfected with Actsol®.

Due to the medical imperative to introduce Actsol® at the earliest feasible opportunity in order to address the nosocomial outbreaks, the use of the solution in the NICU was initiated four months prior to the approval being granted to conduct the full scale trial in all neonatal and surgical wards. The approved study comprised a thorough microbial prevalence screen of all potentially contaminated in-contact surfaces within the NICU and was undertaken in conjunction with the personnel and facilities of the National Health Laboratory, Gaborone, Botswana.

Initial in-vitro antimicrobial efficacy tests with the undiluted ECA solution indicated that a ten minute exposure period was required in order to achieve maximum disinfection. However the prescriptions of the existing cleaning protocols, the workload of the cleaning staff and the need to limit access to the ICU, required that the exposure period to Actsol® to be shortened to five minutes.

While there is no universal agreement as to the recognized sources of nosocomial pathogens, the ubiquitous focus on hand hygiene has presupposed that horizontal transmission is the primary route by which contamination occurs. In the present study, the use of the anolyte solution was restricted to inanimate objects within the NICU (incubators, working surfaces, floors and drains) and no effort was made to include the use of the oxidant solution for the disinfection of the hands of health care workers, nor of the infant patients themselves.

Designated in-contact surfaces within the NICU were sampled by swabbing to enumerate microbial contamination and to identify the prevalence of pathogenic strains that may result in nosocomial infections in the infant patients. The study was carried out over a period of five months and at least 28 sets of samples were collected on three separate occasions from each surface over the study period. Where comparative assessments of antimicrobial efficacy were possible in the post-natal ward, equivalent sized areas within the ward were separated in terms of type of cleaning chemical to be used, and parallel surface samples were collected for each area. The array of cleaning and disinfecting chemicals used in all wards was comprised of chlorine based products, QAC's and the biguanide based formulation, Chlorhexidine. Investigations indicated that the products were rotated on an ad-hoc basis.

8.4.1 Sample Collection and Analyses

Samples were collected from various surfaces by swabbing a 2 x 5 cm (10 cm^2) surface area with a sterile swab dipped in sterile normal saline (0.9% Sodium Chloride). The swabs were then immersed in 10 ml sterile normal saline and transported to the laboratory. In the laboratory, samples were serially diluted and plated out onto three different media; Plate Count Agar (PCA), MacConkey, and Blood Agar (BA). All media were incubated under aerobic conditions at 37^oC for a minimum of 48 hours. On the basis of positive growth on the selective media, suspected pathogenic colonies were isolated and further tested for purposes of identification in accordance with the standard protocols employed by the Microbiology Department of the National Health Laboratory. Microbial enumeration is reported as the Total Viable Count (TVC) and is described by the number of Colony Forming Units per unit sampling area (CFU/cm^2).

The microbial counts for all surfaces sampled were summed and the average microbial count per sampling episode are presented for comparative purposes. No attempt was made to characterize the genotype of the specific strains responsible for the different nosocomial outbreaks and no antibiograms were conducted to establish the antibiotic resistance profile.

8.5 Results

8.5.1 Surface sampling

The results of the initial microbial screen conducted from the various contact surfaces within the NICU and adjacent sluice room, revealed a widespread and consistent degree of contamination by a variety of pathogenic organisms (Table 1). In particular, *Klebsiella spp*. was isolated from most surfaces sampled within the NICU, and displayed a predisposition for moist environments.

This finding accords with the reported partitioning of microbial strains between ‘wet’ and ‘dry’ environments in hospital settings, where ‘dry-type’ sites comprising handles, beds, and curtains were predominantly contaminated by gram positive

organisms, while the 'wet-sites' comprising drains and sinks were contaminated by gram negative bacilli (Gray and Hobbs, 2002; French *et al*., 2004).

Paradoxically, *K. pneumoniae* has been reported to be unstable as an aerosolized fomite at high relative humidity (RH), and displays an enhanced survival and infectious tenacity under conditions of lower RH (Twang *et al*., 2006).

Table 1. Sampling sites and associated microbial pathogens.

Surface	Bacterial strains
Washbasin and Tap lever	*Klebsiella, Pseudomonas, Staphylococcus,*
Floor	*Klebsiella, Staphylococcus, Micrococcus*
Crib	*Staphylococcus, Micrococcus*
Mattress	*Klebsiella, Staphylococcus,*
Shower and Tap lever	*Klebsiella, Pseudomonas, Staphylococcus,*
Toilet Seat	*Klebsiella, Pseudomonas, Staphylococcus, Serratia*

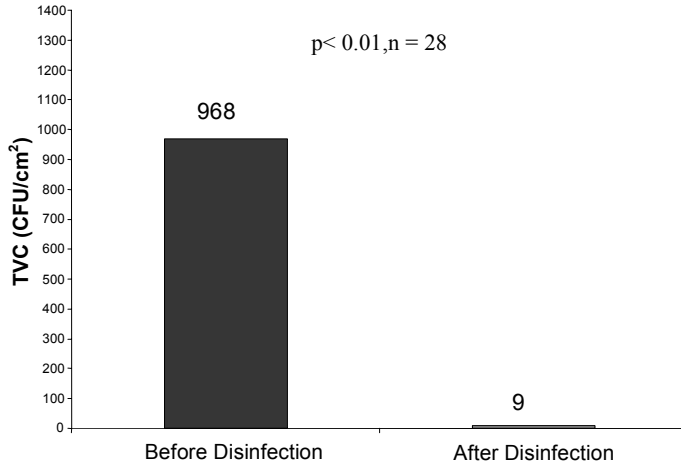
As reported earlier, serious neonatal infections in developing states such as India are predominantly associated with gram negative organisms, and within the NICU, three particular sites were recognized as sources of nosocomial associated pathogens. These comprised of infant incubators and cribs, resuscitation equipment and the various cleaning solutions in use in the facility (Srivastava and Shetty, 2007).

8.5.2 NICU and PNW disinfection

The results of the microbial counts from surfaces in the NICU before and after cleaning with the Actsol® solution clearly demonstrate the antimicrobial efficacy of the oxidant ECA solution when used in a high risk neonatal environment (Fig 1).

The absence of direct comparative data for conventional disinfectant efficacy in the NICU is due to the study being initiated four months subsequent to the first application of Actsol® solution, as was required to address the NICU *Klebsiella spp*. outbreaks referred to earlier.

A direct comparative assessment of the different cleaning efficacies between the standard disinfecting chemicals and Actsol® was only possible in the Post-Natal ward (PNW) and male and female surgical wards. In this aspect of the study, equivalent surfaces were separately disinfected with Actsol® and the standard chemicals.



Legend: TVC – Total Viable Count.

Figure 1. Average NICU surface microbial counts before and after Actsol® disinfection.

The antimicrobial efficacy of Actsol® was further confirmed with the results of the PNW surface swabs, and the substantially persistent microbial load on the surfaces disinfected using standard chemicals clearly indicates a reduced cleaning and disinfection efficacy relative to that of the Actsol® treated surfaces (Fig 2). Disinfection of the NICU with Actsol® reduced the total bacterial count by a magnitude of more than 100 fold (i.e. from an average of 968 to 9 CFU/cm^2). The comparable application of Actsol® in the PNW achieved a 57 fold reduction in the surface microbial count (1488 to 26 CFU/cm^2), while standard chemical disinfection in the PNW achieved an approximately 4 fold reduction (3000 to 733 CFU/cm^2). It is duely recognized that the extensive use of Actsol® in the NICU for four months period prior to the formal study may well have reduced the overall levels of microbial contamination relative to that of the other untreated wards. In addition, the intervention with Actsol®, as well as the extensive sampling and on-site presence may have sensitized the dedicated cleaning staff to the heightened infection control expectations of the trial, and this may further account for the substantive differences in post-cleaning microbial levels between the NICU and the PNW.

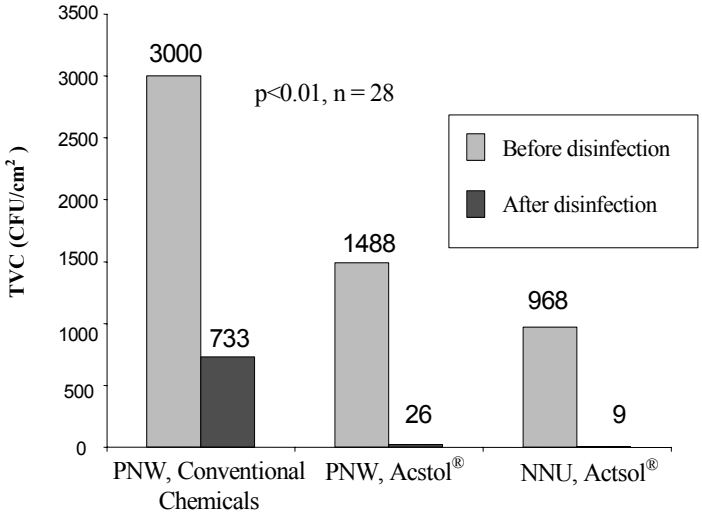


Figure 2. Microbial counts in the NICU and PNW - Comparisons between Actsol® and Standard disinfection.

Anecdotal reports from the nursing staff tended to indicate a higher level of compliance with infection control protocols amongst the cleaners responsible for the NICU relative to those tasked with cleaning the PNW. However, despite these indirect effects, the comparative results for the two disinfectant approaches within the PNW confirmed that the Actsol® solution displayed a substantially increased antimicrobial efficacy as a surface disinfectant relative to that of the standard chemicals employed.

When the results of all three studies are combined, the overall Actsol® antimicrobial efficacy relative to that achieved with the conventional chemical disinfection practices is further substantiated, and the suggested trend of progressive reduction in overall microbial bioload associated with extended Actsol® exposure becomes more tenable.

8.5.3. Extension of the Study to the Surgical Wards

Based on the positive results from the initial phases of NICU and PNW disinfection, the study was extended to the male and female surgical wards to determine whether the observed trends could be duplicated. The same protocol followed within the PNW study phase was adopted, and separate areas were disinfected with the different products. Parallel sampling of equivalent surfaces within each of the two wards was undertaken. Relative to the antimicrobial results obtained in the neonatal wards, the

highly significant differences in antimicrobial efficacy of Actsol® solution relative to that of the standard disinfectants used in the surgical wards suggests that factors other than purely product difference may play a role in justifying the substantive variances (Fig 3).

The equivalent disparity between the results from both the male and female wards confirms the relative superiority of antimicrobial efficacy of Actsol®, but also suggests the role of yet to be determined factors that may account for the difference.

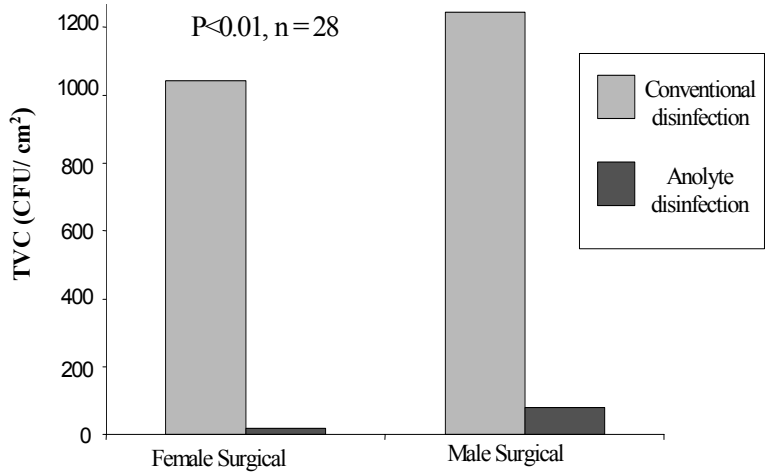


Figure 3. Male and Female Surgical Wards – Comparison between Actsol® and conventional disinfection practices.

8.6 Discussion

The multifactorial aetiology of nosocomial or healthcare associated infections restricts the capacity to accord any single factor as the definitive cause to the event. The dilemma of establishing a casual relationship between impediments in infection control and outbreaks of disease should reflect on the perspective that 'lack of evidence is not evidence of lack' (Griffith, 2006). Given the diverse array of contributing factors, it has been proposed that application of the 'precautionary rule' should be adopted for the resolution of all nosocomial infections (Hambraeus, 2006).

As an extrapolation from the food industry, the precautionary principle states 'where an activity raises the threat to human health or the environment, measures should be

taken, even if the cause-and-effect relationships are not fully scientifically established'(Griffith, 2006).

While neonatal nosocomial infections have been reported to occur from a variety of contaminated sources, it is the infants themselves that are now recognized as being the most frequent reservoir for the horizontal transmission of the pathogens (Casolari *et al*., 2005). This proposal is substantiated by the finding wherein a low level of environmental contamination associated with sporadic enterobacterial disease outbreaks in a neonatal unit, supported the contention that asymptomatic gastrointestinal carriers may be responsible for perpetuating the outbreaks (Gray and Hobbs, 2002; Denton *et al*., 2004).

Other factors that contribute to the risk of nosocomial infections include increased patient turnover and the commercial imperative to have beds filled. Effective manual cleaning and disinfection of complex environments containing beds, furniture and medical equipment is especially difficult under conditions where quick turn-around of facilities are required (French *et al.*, 2004). Furthermore, it has been reported that inappropriate nurse to patient ratios and high patient loads relative to available bed space, all serve to heighten the likelihood of HAI's (Hambraeus, 2006). David *et al*., (2006) have detailed a direct correlation between a high bed occupancy rate, elevated patient to nurse ratios and outbreaks of nosocomial infections in NICU's. These studies are substantiated by the report that heavy clinical workloads and extended working shifts, adversely affected hand decontamination practices and resulted in a concomitant increase in patient infection rates (Chudleigh *et al*., 2005).

A review report from 2006 estimated the annual costs associated with nosocomial infections to be of the order of £1 billion in the UK and up to $25 billion in the USA (Schabrun and Chipchase, 2006). In the USA alone, nosocomial infections are responsible for about 1.7 million infections and 99,000 deaths per year (Curtis, 2008). Aside from the direct cost implications, protracted exposure to largely unwarranted chemotherapeutic agents may selectively promote the evolution of tolerant microbial strains that subsequently become established as sources of life threatening diseases (Dancer *et al*., 2006; David *et al*., 2006).

In all too many instances, the liberal use of both systemic and parenteral broad spectrum antimicrobial compounds are relied upon to afford both a preventative as well as therapeutic cover for inadequate sanitization in the health care environment (Srivastava and Shetty, 2007; Borghesi and Stronati, 2008). This overuse of antibiotics has been proposed as one of the main contributors to resistance development (Chapman, 2003), and it has been reported that up to 70% of clinical isolates from hospitals in the USA, are resistant to at least one antibiotic type (Schabrun and Chipchase, 2006).

Apart from antibiotics, the persistence of infectious surface contaminants has also been associated with the widespread use of the broad spectrum disinfectant, chlorhexidine. This disinfectant has previously been reported to promote the development of resistance (Gray and Hobbs, 2002) and Marrie and Costerton, (1981) have reported on the persistent survival of *Ps. aeruginosa* in a 2% chlorhexidine solution for up to 27 months. This phenomenon has also been reported by Denton *et al*. (2004) who describe iatrogenic cross-contamination with a contaminated Quaternary Ammonium Compound (QAC) based product.

Contrary to exploiting the residual properties of disinfectants, it has been proposed that the inadequate removal of biocidal residues after cleaning, only serves to provide a sublethal adaptive platform for the selection of resistant microbial genotypes (Langsrud *et al*., 2003). In a high risk environment, it is fundamental to ensure that the nominated disinfecting compound is applied at dosages which are relevant to the full array of growth profiles which may be present within the contaminating microbial populations. Persistent environmental contamination is likely to be associated with the presence of established biofilms which may support highly resistant and physiologically distinct microbial populations (Lindsay and von Holy, 2006).

Electro-Chemically Activated (ECA) solutions have only recently been introduced for the purposes of disinfection of high risk medical environments. The results of this study confirm the suitability and appropriateness of expanding the use of this technology within this field. While the precise mechanism of action has yet to be described, the microbicidal effect has been ascribed to the high REDOX potential which results in the destruction of the cell barrier without the need or consequences of

the toxic components normally associated with conventional disinfectants (Marais and Brözel, 1999; Middleton *et al*., 2000; Nakae and Inaba, 2000). A study of the antimicrobial efficacy of the ECA solutions confirms that the synergistic activity of the mixed oxidant constituents substantially reduces the minimum microbicidal or inhibitory concentrations that are required relative to that of the equivalent concentrations of direct chemical analogues (Shimuzu and Sugawara, 1996).

In order to interrupt the well established route of infection transfer by contaminated clothing (Hambraeus, 2006), it is imperative that nursing mothers with infants in the NICU should be educated in the principles of barrier nursing and should be provided with gowns to cover their clothing during their stay within the NICU. The 'kangaroo-care' provided by mothers in developing countries is recognized as a cost-effective and widely accepted style of caring for an infant within hospitals. However the role of this care mechanism and its relationship to the incidence of HAI's from a hygiene perspective remains largely untested (Srivastava and Shetty, 2007). The current arrangement for mothers to nurse their infants during hospitalization and their ready access to the NICU, serves to increase the risk of perpetuating the continuous reintroduction of infectious agents into the NICU.

Discussions with hospital personnel prior to this study confirmed the perception that inadequately sanitized hands remains the single biggest factor for the spread of infections within the hospital. It has been established that there is a strong correlation between homologous hand contamination and the infectious organisms involved in nosocomial infections (Denton *et al.,* 2004). Chudleigh *et al.* (2005) have noted that optimal hand decontamination is considered the most important means of preventing healthcare associated infections, and that the frequency of hand decontamination was substantially less important than the basic hand washing technique in terms of overall disinfection efficiency (Lewis *et al*., 2008). As an adjunct to the surface disinfection study, the microbial contamination of the hands of both mothers and staff associated with the NICU was assessed. Whilst only a superficial screen, the results showed that more than 60% of persons sampled carried pathogenic organisms on their hands after routine washing.

In resource restricted settings, advice on the requirements for optimal hand washing as well as choice of the appropriate antimicrobial agent are substantially more important than the expensive epidemiological assessments that inevitably follow disease outbreaks (Srivastava and Shetty, 2007). Kruglov and Leonov (1999) have reported on the beneficial effects of using the ECA solutions for the disinfection of health care workers hands, and while equivalent in antimicrobial efficacy to 70% alcohol based scrubs, anecdotal evidence suggests that fewer incidents of irritation, skin cracking and dryness with the ECA solutions may translate into enhanced compliance with hand hygiene requirements.

8.7 Conclusions

The near term and sustained reduction of NICU surface microbial counts by the Actsol® solution would suggest that direct contact with contaminated inanimate surfaces was primarily responsible for the original *Klebsiella spp.* infection and that the subsequent cross-contamination of other infants was due to a secondary horizontal hand and/or equipment based transmission. While the infectious focus of the *Klebsiella spp.* appears to have been restricted to nurse's hands and the drains in the sluice room adjacent to the NICU, it is also feasible that the traditional 'kangaroo-care' style of maternal nursing practiced in the NICU may also have perpetuated the inadvertent pathogen transmission by the mothers during their frequent visits into the NICU.

In a resource poor environment it is recognized that a well structured and workable infection control strategy is essential, but that it must have secured universal 'buy-in' in order for it to be sustainable. In developing countries, the most common route for the spread of neonatal nosocomial pathogens is person-to-person transmission within the unit, and the most common iatrogenic factor contributing to neonatal HAI's remains the hands of healthcare workers. Resources, experience, understanding of procedures and commitment toward infection control compliance are recognized as being the fundamental criteria for the control of nosocomial infections in Neonatal Intensive Care units.

The use of Actsol® as a safe and effective surface disinfectant in high risk intensive care facilities was confirmed. On the basis of the positive comparative antimicrobial results, it is proposed that it may readily be used as an alternative to standard disinfecting practices.

8.8 Acknowledgements

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Chapter 9

Antimicrobial efficacy of Actsol®[1], an Electro-Chemically Activated (ECA) oxidant solution against multi-drug resistant bacteria.

9.1 Abstract

The antimicrobial efficacy of Actsol®, an Electrochemically Activated (ECA) oxidant disinfectant produced by Radical Waters, South Africa, was evaluated against a range of hospital Multi-Drug Resistant (MDR) bacterial isolates using a standard *in-vitro* suspension method. The product was tested both with and without the addition of 1% horse serum to evaluate the anti-oxidant effects of bio-soiling. Clinical isolates of MDR bacteria were obtained from the National Health Laboratory Services (N.H.L.S) Microbiology Laboratory at the Chris Hani Baragwanath Hospital, Gauteng, South Africa. These isolates comprised both Gram-positive and Gram-negative bacteria with variable antibiotic resistance profiles, and all strains were recognised as being common nosocomial pathogens within the hospital environment. This study confirmed the excellent broad spectrum bactericidal properties of Actsol® even in the presence of bio-soiling. A marginal reduction in biocidal efficacy was observed when the diluted Actsol® solutions were tested in conjunction with 1% horse serum. There did not appear to be any relationship between the antibiotic resistance profile of the various strains of bacteria and susceptibility to the Actsol® solutions.

9.2 Introduction

The HIV/AIDS pandemic in Africa is placing an ever-increasing burden on the continent's health services. This can be characterised as an increase in the number of patients attending and sojourning in already overcrowded hospitals, a prolonged treatment time, multiple concurrent infections particularly with micro-organisms previously considered as having a low pathogenicity and an increased susceptibility of patients to nosocomial infections where a lower infective dose is required to cause patent clinical disease.

1- Actsol® is the registered trademark of Radical Waters (Pty) Ltd.

In addition to these exaggerated epidemiological pressures, is the concern that while disinfectants remain a cornerstone of any infection control policy designed to limit the spread of pathogenic microorganisms, recent developments have suggested the evolution of co-resistance between disinfectants and antibiotics, where the injudicious use of disinfectants may progressively increase the prevalence of pathogenic MDR strains (Suller and Russell, 1999).

It has been reported that there is a general increase in the number of multi-drug resistant organisms being isolated world-wide (National Nosocomial Infections Surveillance System, 1999). This problem is exacerbated where patients require protracted hospitalisation or where multiple concurrent disease conditions and diminished immunocompetence result in a suboptimal clinical response to chemotherapeutic interventions. This progressive accumulation of MDR strains also significantly increases the risks to attendant health care workers.

A generalised association between antibiotic resistance and reduced susceptibility to disinfectants has been recently reported (Wisplinghoff *et al*., 2007). While reports on chlorhexidine resistance has been shown ever increasingly to be associated with both Methicillin Resistant *Staphylococcus aureus* (MRSA) and Vancomycin Resistant *Enterococcus* (VRE), the authors concluded that resistance to concurrently used disinfectants was not a risk factor in the spread of nosocomial infections and that all the commonly used disinfectants assessed were able to inhibit growth of MDR strains at the recommended concentrations and exposure times.

While resistance to disinfectants was originally ascribed to intrinsic chromosomally encoded responses, extra-chromosomal, plasmid encoded resistance to non-antibiotic agents is now a widely reported phenomenon and specific studies have linked the presence of resistance to chlorhexidine and Quaternary Ammonium Compounds (QACs) to the repeatable presence of plasmids (Russell, 1997).

Resistance to disinfectants presents mainly as an increase in the Minimum Inhibitory Concentration (MIC) required for optimal inactivation, and reduced biocidal sensitivity of Epidemic MRSA or EMRSA strains has been reported to display a 30-

50 fold increase in the MIC of sodium hypochlorite relative to that of Methicillin Sensitive *Staphylococcus aureus* (MSSA) strains (Mycock, 1985).

A diverse array of bacterial tolerances to disinfecting agents has been reported. Aldehyde and biguanide based formulations and the QACs have been shown to display limited anti-mycobacterial activity while the Anilides have been reported to display limited activity against Gram negative bacteria and fungi. The biguanides (e.g. Chlorhexidine) have been reported to lack sporicidal activity, and the bisphenols and halophenols have been shown to have limited activity against the Pseudomonads, Enterobacteriaceae, non-fermenters and moulds (Suller and Russell, 1999; Putman *et al*., 2000; Loughlin *et al*., 2002; Chapman, 2003). Similarly, peroxygen compounds (e.g. Virkon®) have been shown to display limited fungicidal and sporicidal activity, and only exhibit mycobactericidal and virucidal activity in the absence of organic soiling and then only at certain minimum concentrations (Garćia-de-Lomas *et al*., 2008). While there are a limited number of reports suggesting resistance to oxidising agents, a recent study has described an equivalent adaptive reponse to low dose chemically generated hypochlorous acid to that induced by hydrogen peroxide exposure (Mokgatla *et al*., 2002).

However, contrary to these extensive survey data, Wisplinghoff *et al*. (2007) was unable to describe any definitive correlation between antibiotic resistance and decreased disinfectant susceptibility in *Acinetobacter baumannii* at the recommended usage recommendations - this despite widespread suggestions of a directly causal relationship in other gram positive and negative bacterial strains.

Previous reports on the antimicrobial efficacy of Actsol® (Marais and Brözel, 1999; Marais, 2000; Cloete, 2002) or similar ECA based solutions (Selkon *et al*., 1999; Shetty *et al*., 1999; Fenner, 2005) have shown that the product is substantially broad spectrum in activity, as well as being rapidly bactericidal with cell lysis being evidenced within 25 seconds of exposure (Zinkevich *et al*., 2000). Actsol® has been shown to remain stable for up to 6 months under optimal packaging and storage conditions (Radical Waters, Unpublished data). Previous studies conducted in the UK (Selkon *et al*., 1999, Shetty *et al*., 1999) show that it is non- toxic to cells in tissue culture as well as being non-mutagenic (Bakhir *et al*., 2003; Panichev, 2006). *In–vitro*

tests performed by Biocon Research (Pty) Ltd., a South Afrcian laboratory accredited under the guidelines of the FDA, have confirmed that Actsol® is not cytotoxic and non-sensitising to animal cells, in addition to being a highly effective, broad spectrum antimicrobial agent (Marais, 2002).

In an effort to describe a possible causal relationship between MDR and the purported resistance to disinfectants, as well as to reaffirm the different antimicrobial properties between an electrochemically activated oxidant solution and a commercially available hypochlorite preparation, the comparative antibacterial efficacy study was conducted using a variety of confirmed bacterial MDR hospital isolates. To this end, the largely inconclusive reports which to date have suggested a causal relationship between multi-drug resistance and concurrently used disinfectant compounds, has warranted further elucidation specifically with regard to the evaluation of the antimicrobial efficacy of the ECA technology. Additionally, the oxidant neutralisation effect which is conventionally associated with the presence of organic soiling, and the consequential impact on the potential development of resistance to the ECA solutions was also included in the assessment.

9.3 Materials and Methods

9.3.1 Description of Actsol®

Actsol® is the oxidant component of Electro-Chemically Activated (ECA) water generated from a dilute saline solution that has been passed through a powerful electrical field. It is a highly positively charged, mixed oxidant solution and has been reported to be comprised of predominantly hypochlorous acid, chlorine dioxide, hypochlorite, ozone, hydrogen peroxide, and a variety of metastable radicals including hydroxyl, superoxide and singlet oxygen (Prilutsky and Bakhir, 1997; Bakhir, 1999). The prescribed device configuration and operational parameters required to produce the specific ECA solutions has been extensively described in previous chapters.

9.3.2 Source of bacterial strains

Strains of multidrug-resistant bacteria were obtained from the Microbiology Laboratory of the National Health Laboratory Services (N.H.L.S.) at the Chris Hani Baragwanath Hospital (CHBH) in Gauteng, South Africa. Owing to the non-availability of a vancomycin-resistant *Enterococcus* (VRE) strain from the Hospital wards during the course of this study, an American Type Culture Collection (ATCC) strain was secured from an alternative source. The *Pseudomonas aeruginosa* strain which was not highly resistant, but which had an antibiogram profile representative of strains frequently isolated in the wards was also included.

The bacterial strains evaluated in this study comprised of *Acinetobacter baumannii* (5 strains), *Escherichia coli* (4 strains), *Ps. aeruginosa* (7 strains), *Enterobacter* sp. (1 strain), *Salmonella* sp. (1 strain), *S. isangi* (1 serotype), *Klebsiella* spp. (5 strains), *Staphylococcus aureus* (6 strains), *Enterococcus faecium* (2 strains) and *E. faecalis* (1 strain) (Appendix 1 and 2). The array of bacterial strains were recognised to be representative of the organisms commonly encountered during laboratory isolation, and most isolates were prepared from specimens received from patients from the paediatric and respiratory Intensive Care wards.

The bacterial strains were maintained in semisolid agar (N.H.L.S., South Africa) after laboratory isolation and were sub-cultured no more than twice prior to this study in order to maintain the integrity of the antibiotic resistance profile.

As detailed in Table 3, most gram negative bacilli displayed Extended Spectrum β Lactamase (ESBL) activity as well as concurrent High-Level Aminoglycoside resistance. ESBL Salmonellae isolated from the hospital were shown to belong predominantly to the *S. isangi* serotype. MDR MRSA strains (Table 4) were included due to their capacity to produce both catalase and superoxide dismutase, and it was speculated that these mechanisms may have played a protective role during exposure to the hydrogen peroxide and hydroxyl radicals present in the Actsol® solutions.

Ps. aeruginosa is widely reported to be associated with resistance to disinfectants. This has been documented both within hospital isolates as well as those strains

commonly found in the water treatment industry (Brözel, 1992). This study comprised ward isolates which reflected the full array of antibiotic resistance profiles routinely encountered within the hospital (Table 3). Oxidant disinfectants such as hypochlorite are known to display diminished antimicrobial performance in the presence of organic soiling. The addition of 1 % horse serum (N.H.L.S., Virology Department, Rietfontein, South Africa) to the assessment protocol, was included in an attempt to quantify the degree to which the antimicrobial efficacy of the hypochlorite preparation as well as the Actsol® solution would be compromised by the presence of organic matter.

9.3.3 Test conditions, Exposure time and Neutralisation

Actsol® solution was supplied by Radical Waters (Gauteng, South Africa) and was generated from a 0.25% NaCl stock solution, using a previously described FEM based reactor device. The device was preset with a power rating of 12v and 5A and a flow rate of 350ml/min per FEM reactor unit. The range of physicochemical parameters of the different Actsol® solution used in the assessment are characterised in table 1.

Table 1. Physicochemical parameters of the different solutions used in the antimicrobial efficacy assessment.

Parameter	ORP (mV)	pH	EC (mS/cm)	FAC (mg/L)*
Hard Water	235	9.6	0.71	0
Horse Serum	330	7.2	11.4	0
Jik®	550	10.8	1.09	240**
Jik® + Horse serum	515	1.07	1.17	180
Actsol® solutions				
Undiluted	910	6.7	4.92	170
Undiluted + Horse serum	956	6.7	5.02	170
1:10	460	9.7	1.13	10
1:10 + Horse Serum	462	9.0	1.24	10
1:100	263	9.8	0.74	1
1:100 + Horse serum	240	9.6	0.91	1

Legend: ORP- Oxidation Reduction Potential (milliVolts), EC – Electrical Conductivity (milliSiemens per cm), * - FAC: Estimated Free Available Chlorine (milligram per litre), ** - as per manufacturer recommended dilution.

The antimicrobial efficacy of the Actsol® solution was assessed using both the undiluted solution, as well as 1:10 and 1:100 dilutions using standard hard water (0.15g Calcium Chloride, 0.15g Magnesium Chloride in 1000 ml distilled, deionised water) obtained from the N.H.L.S. as the diluent.

Commercially available sodium hypochlorite (Jik®: 3.5% m/v) was used as the alternative chlorine-based comparison, and was formulated to the recommended working strength (i.e. 240mg/L Free Available Chlorine [FAC]) as advised by the manufacturers (Reckitt-Benckiser, SA). The FAC of all solutions was determined using Merckoquant® Chlor-test kits for the 0-20 and 0-500mg/L concentration ranges (Merck, SA). The sodium hypochlorite solution and the Actsol® were used both undiluted, as well as diluted to a 1:10 and 1:100 strength using standard hard water. A control to determine viability count was included, and the Actsol® solution was replaced with a distilled, deionised hard water control and all tests were performed in duplicate.

The procedure for the preparation of the innocula was in accordance with the standard procedures detailed in the guidelines of the South African Bureau of Standards (SABS) (1999). After extraction from the semi-solid agar, all test cultures were grown on standard Mueller-Hinton agar plates (Difco, Detroit, Michigan, USA) at 37^{o}C for 18 hours. Colonies were collected and suspended in distilled water using a sterile metal spreader to give an approximate concentration of 1.0 x 10^{8}cfu/ml by comparing the opacity with a McFarland's 0.5 $BaCl_2$ opacity standard (Koneman *et al*., 1997). A 0.5 ml aliquot of the suspension of each organism was added to 4.5 ml of either the Actsol® or sodium hypochlorite test solution in a sterile screw top plastic test tube, therein yielding a final bacterial load of 1 x 10^{7} cfu /ml.

All test organisms were exposed to the array of biocide solutions for 5 minutes. Where the 1.0% horse serum was included, the serum was first added to the Actsol® solutions, and then thoroughly agitated prior to the addition of the challenge dose of the different bacterial strains. This step was followed in light of the previously reported rapid bactericidal action of the Actsol® solution which may have biased the interpretation of any soil neutralisation effect had it been added after the bacterial challenge (Zinkevich *et al.*, 2000).

Upon termination of the exposure period, the tubes were rapidly inverted 3 times to ensure homogenous distribution of all test organisms. Thereafter, 0.5 ml of the suspension in each of the test solution permutations was removed and added to 4.5 ml of a 1% (m/v) sodium thiosulphate solution, and the tube was inverted several times for a 1 minute period to ensure neutralisation of any residual disinfecting agent. This served to effect a further 10 fold dilution to the challenge aliquot. After the exposure to sodium thiosulphate, a 1 ml aliquot was removed and spread on the surface of Mueller-Hinton agar plates with a sterile metal spreader. All tests were carried out in triplicate and the plates were then allowed to dry for 1 hour before transfer to the incubator. All plates were incubated at 37^{o}C for 48 hours. After incubation, all plates with discrete colonies were enumerated and the mean counts of the colony forming units (CFU) were recorded.

9.4 Results

As detailed in Table 2, the undiluted as well as the 1:10 Actsol® dilution inactivated all organisms within the 5 minute exposure period in the absence of horse serum. The 1:100 Actsol® dilution inactivated all organisms with the exception of the mucoid *Ps. aeruginosa* strains where a 6 $\log_{10}$ reduction was achieved. A direct comparison with the results of the exposure of the bacteria to the commercial sodium hypochlorite solution without the addition of horse serum revealed a substantially equivalent antimicrobial efficacy.

While the addition of horse serum to the diluted Actsol® solutions did reduce the overall inactivation efficiency, the undiluted Actsol® solution mixed with 1% horse serum still achieved a complete inactivation rate despite the presence of the simulated biosoiling. The highly mucoid strains of *Ps. aeruginosa* were shown to display the greatest measure of tolerance to the diluted and soiled Actsol® solutions. Both the undiluted Actsol® and the working strength Jik achieved a 7 $\log_{10}$ kill against these mucoid strains.

Organisms producing catalase (i.e. *S. aureus*) did not display any diminished inactivation when compared against the catalase-negative *Enterococci*, despite the presence of reactive hydroperoxy radicals in the Actsol® solution. Gram negative

bacilli appeared to display greater overall sensitivity to the Actsol® solutions relative to the gram positive coccal strains.

Table 2. Average reduction in microbial log count after exposure to various Actsol® dilutions and sodium hypochlorite both with and without horse serum.

Disinfectant and dilution series	Actsol®			Actsol® + HS			Jik®	Jik®+HS
	100%	10%	1%	100%	10%	1%	As recommended	
Gram negative bacterial strains								
A.baumannii	100	100	100	100	99.99	99.0	100	100
Ps.aeruginosa	100	100	100	100	99.99	99.9	100	100
E.coli	100	100	100	100	100	99.9	100	100
Enterobacter spp	100	100	100	100	100	99.9	100	100
Klebsiella spp.	100	100	100	100	99.999	99.9	100	100
Salmonella spp	100	100	100	100	99.9	99.9	100	100
Gram positive bacterial strains								
E. faecalis	100	100	100	100	99.9	99.0	100	100
E. faecium	100	100	100	100	99.9	97.0	100	100
S. aureus	100	100	100	100	99.9	99.0	100	100

Legend: HS – 1% Horse serum,

9.5 Discussion

Resistance refers to the condition where a bacterial strain is not killed or inhibited by a concentration of biocidal agent to which the strains of that same organism had previously been shown to be susceptible (Russell, 2001).

The bacterial cell is comprised of three distinct components - the genome, the cytoplasm and the outer barrier structure (Brözel, 1992). Of these three, it is widely recognised that the integrity of the barrier is fundamental to the survival of the cell, and that its specific composition and structure confers distinctive abilities to counter the adverse effects of diverse biocidal challenges. Additionally the stage of growth, participation in a biofilm consortium, changes in nutrient availability as well as temperature have all been shown to influence the relative proportions of the different constituents of the cell wall or membrane. This will in turn, directly influence the

barrier properties of the same, and consequently, the ability of the given bacterium to withstand the potentially adverse impact of physical and chemical agents.

Fundamentally, the condition of resistance arises due to either an insufficient quantity of the biocidal agent being presented at the target site, or the increased capacity of the bacteria to externalise or degrade noxious agents and to repair the damage that they may inflict. Resistant states have been reported to arise from a reduced uptake of the antibacterial agent, extracellular neutralisation of the biocide by the presence of the superficial alginate, an increase in cytoplasmic degradatory enzymes, a change in the biocide target site to a non-susceptible state and the induction of efflux mechanisms that cause the expulsion of the noxious chemical agents (Brözel, 1992; Russell, 1997; Cloete, 2003).

Given that neutral ECA anolyte is predominantly comprised of hypochlorous acid, the report by Mokgatla *et al*., (2002) wherein a *Salmonella* isolate was shown to respond to a low dose hypochlorous acid exposure within 10 minutes by inducing increased catalase production is the first indication of potential resistance to ECA solutions. Hypochlorous acid is reported to be a highly destructive and non-selective oxidant that readily reacts with a variety of subcellular compounds to disrupt metabolic processes (Dukan and Touati, 1996). That the low dose hypochlorous acid had been reported to cause severe and progressive DNA disruption within 3 minutes of exposure, would support the suggestion that the autocidal generation of secondary ROS was responsible for the consequential disruption arising from the initial insult. Additionally, it was shown that the increased levels of catalase activity in the hypochlorous acid tolerant strain resulted in a two fold increase in the degradation of hydrogen peroxide relative to that of the hypochlorous acid sensitive strain. This response would suggest that there may have been a non-specific induction of an intrinsic adaptive mechanism which was geared primarily to a cellular reponse normally mediated by hydrogen peroxide. It must be emphasised that the hypochlorous acid used in this study was not generated by an electrolytic process and hence did not have the synergistic biocidal benefits of the elevated REDOX potential previously described (Prilutsky and Bakhir, 1997, Bakhir, 1999, Marais, 1999; Zinkevich, 2000). In the absence of an elevated REDOX exposure, it is thus proposed that the resistant isolate was able to protect itself against exposure by decreasing the

levels of reactive species which could be expected to react with hypochlorous acid to generate toxic reactive oxygen radicals, and that the mechanism of oxidative tolerance was due to a combination of physiological adaptations which collectively led to an enhanced degree of tolerance (Mokgatla *et al*., 2002).

The role of extracellular barrier mechanisms to neutralise the non-selective and highly reactive oxidant species in the Actsol® solutions is supported by the enhanced sensitivity displayed by the non-mucoid strain of *P. aeruginosa* relative to that of the two mucoid isolates when tested at a 1:10 dilution in the presence of horse serum. This differential biocidal effect was shown to be independent of the antibiotic resistance profile of the different strains, as all isolates displayed a substantially equivalent MDR profile. It is thus proposed that the oxidant chloroxy and hydroperoxy radicals of the Actsol® solutions may have reacted with the alginate of the mucoid *Ps. aeruginosa* strains and that the latter acted as a sacrificial antioxidant to the mixed oxidants in the Actsol® solution, thereby leaving the core underlying cell structures intact.

While it is acknowledged that certain correlates may be drawn between MIC derived disinfectant resistance profiles and the presence of specific genes that have been reported to encode for the efflux of the same categories of biocidal agents, the limited conclusions drawn may not necessarily reflect the broader and more holistic dictates that govern the survival of a biocide challenged microorganism.

Commentary on the validity of the use of manufacturer MIC's as definitive descriptors of states of tolerance or resistance have been questioned, and it has been suggested that laboratory based resistance determinations may, at best, be tenuous. Relative to the selection pressure exerted by intensive antibiotic use and the consequential selection of adaptive geno- and phenotypes, the claims of purported resistance to disinfectants may be viewed as trivial, and it has been proposed that the rate of inactivation rather than the degree of inhibition is a substantially more relevant indicator of clinical bacterial susceptibility to disinfectants (Suller and Russell, 1999). Despite the vast number of reports that suggest otherwise, the efflux of a broad range of structurally unrelated toxic compounds may also be viewed as a consequence of a

normal primary physiological function and thus a fortuitous side effect of the transport of a common physiological or metabolic substrate (Putman *et al*., 2000).

Aside from its proven antibacterial efficacy, direct exposure to Actsol® solutions has shown that it is non-cytotoxic to skin, mucous membranes and the conjunctiva, and that it does not precipitate skin hypersensitivity reactions in guinea pigs, rats or rabbits (Marais, 2002; Bakhir *et al*., 2003). It has been proposed that the substantially safer exposure profile is due to the action of low levels of hypochlorous acid (HOCl) which closely mimics the mechanisms of the mammalian leucocyte based antimicrobial system (Cunningham and Ahern, 1995).

In the undiluted state, reasonable levels of soiling (equivalent to 1% horse serum) did not impair the antimicrobial efficiency of the electrochemically activated Actsol® solution. Additionally, the antimicrobial efficacy of the undiluted Actsol® solution was shown to be directly equivalent to the commercial hypochlorite solution, albeit that the FAC concentration of the two solutions was 170 and 240mg/L respectively.

The choice of standard hard water as a diluent for the oxidant Actsol® solution resulted a substantial reduction in the ORP of the diluted solutions. This is attributed to the elevation in pH and the conversion of the available chlorine into the less microbicidal hypochlorite moiety which may have limited the antimicrobial efficacy of the diluted Actsol® solutions. It is proposed that the reduction in the REDOX potential without any significant change in FAC concentration was primarily responsible for the substantial reduction in antimicrobial efficacy.

9.6 Conclusions

This preliminary study indicates that Actsol® could be a useful disinfecting agent for reducing the incidence of nosocomial outbreaks in health care facilities, and hence, is an effective tool to manage the development of multi-drug resistant strains commonly associated with these persistent nosocomial infections. A rapid bactericidal effect against both gram-positive and negative MRD bacterial strains was achieved even in the presence of simulated biosoiling

Despite a substantially lower active reagent concentration (FAC), the neutral anolyte was able to achieve an equivalent degree of bacterial inactivation to that of the commercial hypochlorite over the same exposure periods. Both the electrolysed and chemical oxidant solutions were able to inactivate all strains of bacteria irrespective of their antibiotic resistance profiles and the results would suggest that there is no cross or co-resistance between the encoded antibiotic resistance and the oxidant species present in the two test solutions.

These results confirm that Actsol® is an effective alternative biocidal agent against most bacteria including MDR strains and that it retains its antibacterial efficacy in the presence of low levels of organic material.

9.7 Acknowledgements

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9.10 Appendices

Appendix 1. Antibiotic resistance profile of gram negative bacterial isolates evaluated in the study.

Gram Negative Organisms	Imperilling	Co-amoxyclav.	Piperacillin	Pip-taz	Cefazolin	Cefuroxime	Ceftriaxone	Ceftazidime	Cefepime	Imipenem	Meropenem	Gentamicin	Amikacin	Tobramycin	Ciprofloxacin	Cotrimoxazole	Chloramphinicol
P.aeruginosa (M)			s	s				s	s	r			s		s		
P.aeruginosa (M)			r	r				r	r	r	r	r	r		r	r	r
P.aeruginosa			s	s				s	s	s	s	r	s		s	r	r
P.aeruginosa			r	r				s	s	s	s	r	r		r	r	
P.aeruginosa			r	r				r	r	r	r	r	r		r	r	s
P.aeruginosa			s	s				r	s	r	r	r	r		r	r	r
P.aeruginosa			r	r				r	r	r	r	r	r		r	r	r
A.baumannii	r	r	r	r	r	r	r	r	r	r	r	r	r	s	r	r	
A.baumannii	r	r	r	r	r	r	r	r	r	s	s	r	r	r	r	r	
A.baumannii	r	r	r	r	r	r	r	r	p	s	s	r	r	r	r	r	
A.baumannii	r	r	r	r	r	r	r	r	r	r	r	r	r	s	r	r	
A.baumannii	r	r	r	r	r	r	r	r	r	r	r	r	r	p	r	r	
A.baumannii			r	r				r	r	r	r		r	s	r		
A.baumannii			r	r				r	r	s	s		r	r	r		
S. isangi	r	r		s			r			s					s	r	
Salmonella sp.	r	r	r	r	r	r	r	r	r	s	s	r	s	r	s	r	
Enterobacter sp.	r	r	r	r	r	r	r	r	r	s	s	r	r	r	s	s	
E.coli	r	r	r	s	r	r	r	r	r	s	s	r	s	r	s	r	
E.coli	r	r	r	s	r	r	r	r	r	s	s	r	s	r	s	r	
Klebsiella sp.	r	r	r	r	r	r	r	r	r	s	s	r	s	r	s	s	
Klebsiella sp.	r	r	r	s	r	r	r	r	r	s	s	r	s	r	s	r	
Klebsiella sp.	r	r	r	p	r	r	r	r	r	s	s	p	r	r	s	r	
Klebsiella sp.	r	r	r	s	r	r	r	r	r	s	s	r	s	r	s	r	
Klebsiella sp.	r	r	r	r	r	r	r	r	r	s	s	r	s	r	s	r	

Legend: r – Resistant; s – Sensitive; p – partially resistant ; (M) – Mucoid.
(Resistance type: *A. baumannii* – ESBL and AME; *E coli* –ESBL; *S. isangi* – ESBL; *Enterobacter* sp. – ESBL; *Klebsiella* spp – ESBL)

Appendix 2. Antibiotic resistance profile for gram positive bacterial isolates evaluated in the study.

Gram positive Organisms	Pen/Ampicillin	Oxacillin	Erythromycin	Clindamycin	Tetracycline	Co-trimoxazole	Rifampicin	Vancomycin	Fucidin	Ciprofloxacin	Chloramphenicol	Gentamycin high level conc.
MRSA	r	r	r	s		r	s	s	s	r		
MRSA	r	r	s	s		r	r	s	s	s		
MRSA	r	r	r	s		r	r	s	s	s		
MRSA	r	r	r	r		r	r	s	s	r		
E. faecium	r	r	r	r	s	r	r	s	s	r	r	r

Legend: r – Resistant; s – Sensitive.