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An Investigation into Pre-Surgical Skin Preparation Techniques and the Role of Chlorhexidine Resistance in the Presence of Flora in Canine Patients

Nicola Swales BSc(hons) RVN^A and Dr Tristan Cogan BSc(Exon), PhD(Exon)^B

A. Small Animal Hospital, Langford Vets, University of Bristol, Bristol, UK

B. Microbiology Department, University of Bristol, Bristol, UK.

Correspondence: Nicola Swales, Small Animal Hospital, Langford House, Langford, Bristol, BS40 5DU. nicola.swales@bristol.ac.uk

Abstract

Skin swab samples were taken before and after skin preparation in 25 dogs undergoing abdominal surgery. Culture and sensitivity testing was undertaken. Eight out of 25 dogs had bacteria present on the skin after preparation, of which three were prepared using the concentric circle and five using the linear method. 100% of isolates were resistant to at least one antibiotic and three were resistant to Chlorhexidine. There was no significant difference in the efficacies of either method. Chlorhexidine resistance plays a significant role in the presence of post-preparation bacteria.

Keywords: Skin preparation, Chlorhexidine, Surgical Site Infections, Resistance, Commensal Flora

There are two commonly-used methods of pre-surgical skin preparation of canine patients; the linear method, with a 'back and forth' motion, and the concentric circle method; which starts in the centre, working outwards towards the limits of the surgical site.

The incidence of Surgical Site Infections (SSIs) in both human and veterinary patients is significant, with studies showing they occur in at least 15% of elective human patients (Dumville, Mcfarlane, Edwards, Lipp & Holmes, 2004; Mangram, Horan, Pearson, Silver & Jarvis, 1999). SSIs occur when the bacterial load overcomes the host's immune defences; when there are between 10^5 and 10^6 bacteria per gram of tissue (Mangram et al., 1999). The aim of skin preparation is to reduce the risk by removing 'soil and transient organisms', to decrease resident flora to below the threshold, and to inhibit regrowth (Bowers, 2012; Dumville et al., 2004).

Skin flora

The integument has millions of bacteria that penetrate the dermal layers, hair follicles and sebaceous glands (Price, 1938). Veterinary patients have high numbers as they do not wash like humans, are kept in dirtier environments, and have more hair follicles. Animals are more likely to become contaminated via the oral-faecal route and to spread bacteria during grooming. Commensal bacteria fall under two categories, determined by their attachment.

Transient flora

Transient flora are not part of normal skin flora. They are acquired through contact with people, animals and contaminated fomites (Dingle & Rock, 2006), and can be transferred similarly. They are easily removed with slight friction and are usually completely removed by biocides. They inhabit only the skin surface (Bowers, 2012; Dingle and Rock, 2006).

Resident Flora

The population size and composition of resident flora is more constant. They are adhered firmly to the skin (Price, 1938) and up to 20% are sequestered deeper in the stratum corneum, and in follicles and glands (Warner, 1988). Resident flora are therefore harder to remove. Complete sterilisation of the skin is would cause significant trauma so the aim of skin preparation is to decrease the population to be under the threshold for infection (Price, 1938; Warner, 1988).

The most common resident bacteria in veterinary patients are the Gram-negative *Acinetobacter baumannii* (Boerlin, Eugster, Gaschen, Straub and Schawalder, 2001), *Escherichia coli* (*E. coli*), *Klebsiella spp.*, *Pseudomonas aeruginosa*, and the Gram-positive *Staphylococcus spp.* (Warner, 1988).

Biofilms

A biofilm is a complex consortium that results from the association of bacteria with solid surfaces (Russell, 1999). They respond poorly to biocides due to reduced penetration (Russell, 2001). This is concerning as around 90% of bacteria can reside in biofilms and no surface has been found that cannot be colonised; including implants, sutures, and catheters (Bonez et al., 2013). Studies have shown that the minimum inhibitory concentration (MIC) of biocides can be 1000 times higher for bacteria in biofilms (Davies et al., 1998; Donlan, 2001) compared to those for free-floating bacteria (Madigan, Martinko, Dunlap & Clark, 2009).

Biocides

Biocides for skin preparation need to be bactericidal, fungicidal, virucidal and sporicidal, with a residual action that slows or prevents regrowth. They must be non-toxic and quick to apply and dry, to not to prolong the anaesthesia time, and cause minimal hypothermia (Gibson, Donald, Hariharan & McCarville, 1997).

The most commonly used biocides are Povidone-Iodine (PI) and Chlorhexidine (CHX). Studies have shown CHX to be superior in reducing the amount of bacteria and incidence of SSIs (Berry, Watt, Goldacre, Thomson & McNair, 1982; Darouiche et al., 2010; Mimos et al., 2007; Noorani, Rabey, Walsh & Davies, 2010). CHX is a positively-charged molecule (Jones, 1997) that binds to negatively-charged molecules in cell walls (Nittayananta et al., 2008) in order to enter (Kõljalg, Naaber & Mikelsaar, 2002). It is deactivated by organic matter and is more effective at alkaline pH (Russell & Path, 1986). Studies have shown a rising prevalence of CHX resistance.

Chlorhexidine Resistance

The term ‘resistance’ means bacteria are not destroyed or inhibited by commonly-used biocidal concentrations, that similar organisms are susceptible to (Russell, 2000). Bacterial spores are the most resistant, with mycobacteria and gram-negative bacteria, then gram-positive bacteria following. There is also variation between different species, such as *Pseudomonas spp.* and *Proteus spp.* being more resistant than other gram-negative bacteria (Russell, 2001).

Intrinsic Resistance

Uptake of biocides occurs by diffusion (Russell, 1999). Intrinsic resistance is an innate property that causes impermeability, such as the lipid-rich membrane of mycobacteria (Russell, 2000) or in Gram-negative bacteria, where the high magnesium content causes strong bonds, preventing uptake (Russell, 2001). Diffusion of biocides can be impeded by loss or alteration of porins, while some bacteria have the ability to degrade biocides and others have efflux pumps. Bacteria that have hydrophobic membranes (Maillard, 2007) and those that reside within biofilms have increased resistance (Poole, 2002; Russell, 2001).

Acquired Resistance

Acquired resistance is a result of genetic mutation, plasmid acquisition (Russell, 2000), or non-plasmid resistance; an unstable mechanism (Maillard, 2007) where bacteria are 'trained' in sub-inhibitory concentrations of biocide and can thus withstand gradual increases (Russell, 1999). Plasmid resistance is when fragments of DNA (Madigan et al., 2009) are transferred between bacteria, for example the *qac* genes found in methicillin-resistant *Staphylococcus aureus* (MRSA) (Poole, 2002; Russell, 2001) which encode efflux pumps (Sekavec, Moore & Gillock, 2013). An example of mutation is present in the *mar* genes found in *E.coli*. A mutation in the regulatory sequence causes altered expression of genes, including overexpression of *acrAB* which encodes an efflux pump (Levy, 2002; Russell, 2000). In the clinical setting, not only does resistance pose a threat by increasing the risk of SSIs, we may also be selecting for resistant bacteria (Russell, 2000).

Surgical Site Preparation

Though contraindicated in humans (Mangram et al., 1999), the first stage of skin preparation in veterinary patients is hair removal. Clipping can cause microscopic cuts that allow resident bacteria to surface, but is necessary to prevent hair contamination. Clipping also enables visibility to check for infection which would necessitate postponing elective surgery. Care should be taken to not cause trauma; the clippers should be clean, lubricated, and well-maintained (Bowers, 2012).

Once the hair has been removed, the primary scrub takes place. The purpose is to remove dirt, debris and transient bacteria. To do this, a combination of biocides and mechanical cleansing is needed. The vigour with which one scrubs is important as friction is needed to remove tightly-adhered resident flora (Bowers, 2012; Price, 1938). Because some resident flora live

deep in the skin (Roberts, 2013), abrasions could cause recontamination (Bowers, 2012, Warner, 1988).

Studies have shown that temperature does not alter efficacy (Price, 1938) however a warmed solution of 50:50 CHX with water should be used to prevent hypothermia (Roberts, 2013). Historically, the preferred scrub method has been to start at the proposed incision site and work out towards the margins of the clipped site in concentric circles (Mangram et al, 1999; Roberts, 2013). Anecdotal evidence states that it prevents the incision site being recontaminated by marginal areas. There does not appear to be much evidence for this (Bowers, 2012) and some suggest that this method does not allow adequate penetration into fissures in the skin. Others have shown the linear method to be more efficacious (Roberts, 2013) while some say a combination should be used (Crosby, 2000). Non-lint-producing cotton swabs should be utilised to prevent fibre contamination, swabs should be discarded regularly and the scrub should last for the minimum contact time to provide residual action (Bowers, 2012; Roberts, 2013), to reduce rebound growth, as resident flora tend to repopulate quicker than the original colonisation (Bowers, 2012).

To preserve cleanliness, the surgical site should be covered before moving into theatre where a sterile scrub is performed. Sterile gloves and sterile swabs or applicators used to perform a similar scrub, adhering to the contact time. Some prefer a CHX paint rather than a scrub (Masterson, 1988) that has increased residual action (Bowers, 2012).

Method

Ethical approval for this study was acquired from the University of Bristol's Research Ethics Committee. The subjects were 25 client-owned dogs that were to undergo abdominal surgery.

No selection limitations or exclusion factors were placed on age, breed, or sex. Patients had hair clipped from the thoracic outlet to the pubic symphysis, and to the lateral skin edges.

Data Collection

A nylon-flocked swab (eSwab™, Copan Diagnostics, USA) was used to collect a sample of skin flora from the abdominal midline, after clipping. The swab was rubbed for five seconds then stored aseptically. Non-woven swabs (Sofsborb™, Synergy Health, UK) were soaked in a 50:50 solution of Chlorhexidine (Vetasept® Chlorhexidine, Animalcare, UK) and skin scrub methods were alternated between the linear method and concentric circle method. The skin scrub was ceased after five minutes, if a dry swab appeared visibly clean after rubbing across the midline and each outer edge of the clipped site, or was continued until such time that a swab did appear clean. A 'post-scrub' flora sample was taken using the method previously described. The author performed all sampling and skin preparation to minimise variation.

Culturing

100µl of the liquid from each swab container was cultured on MacConkey Agar with Salt (MAC) and Mannitol Salt Agar (MSA) (Thermo Fisher Scientific, UK). The plates were incubated for 24 hours at 37°C after which the number of colonies was counted. For ease, plates with an excess of 200 colonies were recorded as 200. Colonies from post-preparation swabs were sub-cultured onto Blood Agar (BA) (Thermo Fisher Scientific, UK), to allow greater proliferation.

Biochemical Tests

Tests were undertaken to identify the bacteria. Hydrogen Peroxide (Sigma, USA) was used to test for catalase production, urea broth (Thermo Fisher Scientific, UK) for urease-producing

bacteria (Madigan et al., 2009) and triple sugar iron (TSI) agar slopes were inoculated on the surface and in the body of the agar to distinguish between aerobic and anaerobic species.

To test for sensitivity, a small sample of each bacteria was suspended in 5mls of 0.9% Phosphate Buffered Saline then spread on BA. After drying, discs infused with amoxicillin and clavulanic acid (AMC), cefovecin, (CVN), ceftazidime (CAZ), sulphamethoxazole *?????* (RL), and cefpodoxime and clavulanic acid (CD) (Oxoid, UK) were placed on the surface. After incubation, the diameter of the halo, if present, was measured and compared to guidelines for the determination of resistance (<http://bsac.org.uk/susceptibility/>). To test for CHX resistance, BA plates were inoculated then submerged in 4mls of 50:50 CHX and distilled water. The plates were incubated overnight then observed for growth.

The MIC was found for each isolate by first culturing in Brain Heart Infusion Broth (Oxoid, UK) and incubating, before performing serial dilutions, using a standard microdilution method (<http://clsi.org/>), to determine bacteriostatic concentrations. After incubation, samples were cultured onto BA to determine the presence of live bacteria and the bactericidal concentration.

Results and Statistics

Excel (Microsoft, USA) was used to record results and Prism (GraphPad Software, USA) was used for statistical analysis and graphs. A paired Wilcoxon Signed Rank test, a non-paired Mann Whitney U test, and a one-tailed Fisher's Exact test were used for statistical analysis. Significance is defined as having a 'p' value less than 0.05.

Results

Of the 25 dogs sampled, eight (32%) had bacteria present after preparation. Both preparation methods significantly decreased the incidence of bacteria, with p values of 0.0056 (MAC) and

<0.0001 (MSA) for the linear method, and 0.0014 (MAC) and <0.0001 (MSA) for the concentric circle method. The decrease in prevalence was significant, with an average decrease of 99% (see figure one) and p values of 0.0012 (MAC) (see figure two) and 0.0008 (MSA) (see figure three) for the linear method and average decreases of 89% (MAC) and 99.9% (MSA) (see figure one) and p values of 0.0029 (MAC) (see figure four) and 0.0002 (MSA) (see figure five) for the concentric circle method.

Of the eight dogs, three (37.5%) were prepared using the concentric circle method and five (62.5%) using the linear method. There was no statistical significance between the incidence of bacteria (p= 0.3867 and 0.7400 for MAC and MSA respectively) and no significance between the prevalence (p=0.36 (MAC) (see figure six) and p=0.48 (MSA) (see figure seven)).

The bacteria isolated were *E. coli*, *Bacillus spp.*, *Klebsiella spp.*, and *Shigella spp.*. All isolates showed resistance to at least one antimicrobial agent and three out of eight (37.5%) were resistant to CHX, with MICs of 3.125%. Two out of five pre-preparation samples showed evidence of CHX resistance (see figure eight).

Discussion

Clinical Significance

In the majority of cases, SSIs are caused by commensal rather than extraneous flora (Reichman & Greenberg, 2009). The existence of CHX resistance in commensal flora is significant within the veterinary profession as 77% of practices use CHX (Evans, Knowles, Werrett & Holt, 2009). SSIs create an increased economic burden for practice and owner, with increased visits, additional therapies and surgeries, diagnostic procedures and lengthy hospitalisation often being necessary (Nicoll, Singh & Weese, 2014). The welfare of the patient is also at risk as

additional stress and can lead to increased morbidity (Oxlund, Fogdestam & Viidik, 1979). Surgical procedures and anaesthesia always carry risks, especially in compromised patients (Gaynor, Dunlop, Wagner, Wertz, Golden & Demme, 1999) and inflammation leads to pain and poor welfare. The patient may need additional analgesia which is a further cost and can carry risks (King et al., 2011; KuKanich, Bidgood & Knesl, 2011).

CHX resistance also poses a threat to human medicine. A recent study showed that in one hospital, the median increase in hospitalisation length for an SSI was ten days and the median additional cost per patient was over £5000. This represents vast amounts of money lost as the total extra cost for hospital was almost £2.5 million in two years (Jenks, McQuarry & Watkins, 2014).

Though the author only isolated two species of resistant bacteria, other studies have found CHX resistance in *Proteus spp.* (Dance, Pearson, Seal & Lowes, 1987), *Staphylococcus spp.* (Fritz et al., 2013), *Klebsiella spp.* (Evans et al., 2009), and *Pseudomonas spp.* (Levy, 2002).

Pre-surgical Skin Preparation

Anecdotal evidence states that the concentric circle method prevents recontamination while the linear method provides more friction. This additional friction did not lend any advantage, with results showing no significant difference between the methods. Personal preference may therefore be used.

Method of Action

Bacterial cells have three target regions for biocides to act upon; the cell wall, the plasma membrane, and the cytoplasm. Access to these regions is determined by extracellular material, cell morphology, and internal chemical composition (Denyer & Stewart, 1998). The plasma membrane tends to be the focus as biocides have to penetrate it to reach the cytoplasm. It is a

semipermeable phospholipid-membrane that regulates homeostasis by transporting substances in and out of the cell. Disruption can occur by breaking the membrane, preventing active transport processes or inhibition of enzymes required for cellular functions. CHX works by first damaging the cell wall in order to gain access to and adhere to the plasma membrane, causing lysis (Maillard, 2002).

Chlorhexidine Resistance

The presence of CHX resistance could be due to various factors. *Bacillus spp.* are able to produce endospores; when they transform into a non-growing structure, usually due to lacking key nutrients. The structure is different from that of the normal bacterium, with additional non-permeable layers around the cell membrane, known as the exosporium (Madigan et al., 2009; Russell, 2001).

Shigella spp. have a different mechanism of resistance. One such mechanism involves the presence of the *mar* phenotype (Abiola, Olufunke, Benjamin & Bosede, 2014; Cohen, Hachler & Levy, 1993). Upregulation can be caused by external stimuli, such as the use of biocides (Russell, Tattawasart, Maillard & Furr, 1998; Sheldon Jr, 2005).

Bacteria that are more tightly adhered have a greater chance of survival. This could be because they are either sequestered in hair follicles or are part of a biofilm. In biofilms, efflux pumps are not needed and bacteria that are usually susceptible have greatly reduced susceptibility. One mechanism is related to penetration. 'Slow or incomplete penetration', due to aggregation of the organisms to each other and to the dense extracellular matrix (Madigan et al., 2009), could mean that some bacteria survive, allowing recolonisation.

Oxygen gradients within biofilms lead to anaerobic areas and accumulation of acidic waste products. This decreases the pH and can antagonise biocides, as CHX is known to be less

effective in acidic conditions (Russell & Path, 1986). This accumulation could also cause bacteria to enter a spore-like state (Stewart & Costerson, 2001).

Limitations

Cases were limited to patients requiring surgery within a four-week period, meaning a larger sample size was not possible. No selection limitations were applied to patients with regards to antibiotic use which could have caused a reduction in skin flora. This could explain the low number of colonies on pre-preparation samples in some cases. Also, as the skin was vigorously scrubbed, some transient bacteria that were resistant to CHX may have been removed, therefore making it impossible to determine MICs for all resistant bacteria. There was no opportunity to take samples after the sterile skin preparation as this was not performed on all cases.

Within the confines of this study, it was not possible to discover if biofilms were present and thus what role they played in resistance. It was also not possible to recreate these in the laboratory setting and the results of in vitro testing may have differed to in vivo circumstances.

Future research and Improvements

In the future, a larger sample size could confirm these findings and also allow samples after the sterile skin preparation. Effort should be made to test for biofilms and to recreate them in vitro, to see if they have any effect on the incidence of resistance. Selection limitations should prevent inclusion of patients with concurrent skin disease or concomitant antibiotic use. A follow-up looking into the incidence of SSIs would be helpful. New research should be undertaken with respect to alternative biocides, such as Iodine Povacrylex, as some studies have found it more efficacious than CHX (Hemani & Lepor, 2009; Swenson, Hendrick, Metzger, Bonatti, Pruett & Sawyer, 2009). Another biocide with limited research is Parachoroxlylenol, which has been shown to be efficacious (Zinn, Jenkins, Swofford, Harrelson

& McCarter, 2010) and safe for mucous membranes and ears, unlike CHX which can be irritant and is ototoxic (Igarashi & Suzuki, 1985). Further research could be undertaken into blocking efflux pumps biocides that can penetrate biofilms and endospores.

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