Research Article

An invitro Study of 2.5, 5.0, and 10% Providone-Iodine (Betadine) Solutions in Inhibiting the Growth of Different Gram Negative and Gram Positive Pathogens Showed a Lack of Efficacy with Several Bacterial Species

Tran P1, Desai A1, Luth K1, Huynh E1, Dong H1, Dev A1, Hamood A2, Mitchell K1 and Reid T1,2*

1Department of Ophthalmology and Visual Sciences, Texas Tech University Health Sciences Center, Lubbock, TX
2Department of Immunology and Molecular Microbiology, Texas Tech University Health Sciences Center, Lubbock, TX

*Corresponding Author: Dr. Ted W. Reid, Department of Ophthalmology and Visual Sciences, Texas Tech University Health Sciences Center, Lubbock, TX; Tel: 806-743-2020; Fax: 806-743-2471; E-mail: ted.reid@ttuhsc.edu

Published: September 10, 2016

Abstract:

Background: Povidone-iodine (PVP-I; Betadine) solution is a topically applied antiseptic which has many uses in wound care, surgery, pre-operative and post-operative applications for many decades. However, there is evidence that has found contamination of PVP-I solution in many hospitals. This has been thought of as a biofilm problem. Thus the present study was undertaken to measure the effectiveness of different PVP-I solutions in inhibiting the growth of different Gram negative and Gram positive bacteria.

Methods: We examined the antimicrobial activity of 2.5%, 5%, and 10% PVP-I solutions against Staphylococcus aureus, Pseudomonas aeruginosa, Staphylococcus epidermidis, Escherichia coli, Serratia marcescens, Acinetobacter baumannii and Methicillin-resistant S.aureus by both the disc diffusion (zone of inhibition) and the viable count assays.

Results: All the bacteria, except P. aeruginosa, using 2.5% PVP-I, showed zones of inhibition. At 5 and 10% PVP-I concentrations, all the bacteria showed zones of inhibition. The average inhibition zones in mm for Gram positive bacterial group are 3.94, 5.54, and 8.03 for 2.5, 5, and 10% PVP-I respectively. The average inhibition zones in mm for Gram negative bacterial group are 1.56, 3.36, and 5.10 for 2.5, 5, and 10% PVP-I respectively. However, after measuring the inhibition zones, the disks were tested for live bacteria and only Staphylococcus aureus and MRSA strains showed no live bacteria at 10% PVP-I. The confocal laser microscopy confirmed the viable count results.

Conclusions: This study demonstrated the following: 1) the zone of inhibition assay does not give a realistic assessment of the ability of an antimicrobial to kill bacteria; 2) In vitro, 2.5% PVP-I is not effective at killing any of the bacterial species associated with post procedure endophthalmitis; 3) 5% PVP-I is only effective at killing S. marcescens; and 4) 10% PVP-I is only effective against S. aureus, MRSA, and S. marcescens strains, but not P. aeruginosa, S. epidermidis, E. coli, and A. baumannii strains. Thus, the use of PVP-I as a means of removing bacteria is problematic.

Keywords: Antiseptic; Betadine; Povidone-Iodine

Abbreviations: ANOVA: Analysis of Variance; CFU: Colony Forming Unit; CI: Clinical Isolate; CLSM: Confocal Laser Scanning Microscopy; GFP: Green Fluorescent Protein; LB: Luria-Bertani; MH: Mueller Hinton; MRSA: Methicillin-Resistant Staphylococcus Aureus; OD: Optical Density; PBS: Phosphate Buffer Saline; PVP: Polyvinyl Pyrrolidone; PVP-I: Povidone-Iodine; RPM: Round per Minute; SD: Standard Deviation; ZOI: Zone of Inhibition
Introduction: Povidone-iodine (PVP-I) or Betadine is a broad spectrum antiseptic for topical application in the treatment and prevention of wound infection [1-5]. PVP-I is a stable chemical complex of polyvinyl pyrrolidone (povidone, PVP) and elemental iodine, and it contains from 9.0% to 12.0% available iodine, calculated on a dry basis [6]. After its discovery in 1955 by H. A. Shelanski and M. V. Shelanski, it was widely used around the world, to treat wounds [4,7]. PVP-I is a widely used antiseptic because it has a particularly broad spectrum of antimicrobial activity that includes Gram-positive and Gram-negative bacteria, bacterial spores, fungi, protozoa and viruses [4,8-12]. This wide spectrum activity stems from the mechanism of action of PVP-I, it releases iodine (free iodine) which readily penetrates microbial membranes and leads to intra cytoplasmic protein oxidation [4,10,12].

Povidone-iodine is commonly used as a surgical scrub and is applied pre- and post-operative to surgical sites [2,3,5,11,13-21]. Currently, it is generally accepted to use 5% PVP-I solutions to prepare different surfaces such as for intravitreal injections, wound preparation or preoperative cataract surgery [16,22]. The reason concentrations below 5% are not used is because it has been shown that concentrations below 5% are not effective bactericidal agents [22,23].

Injections of intravitreal medications have become routine care in ophthalmology offices throughout the world for the treatment of several retinal diseases. Studies estimate that the rate of endophthalmitis from intraocular injections range from 167 in 10,000 to 6 in 100,000 cases. Currently, 5% povidone-iodine is widely accepted as the main antiseptic used to decrease the risk of endophthalmitis [22]. For surgical use, the American Academy of Ophthalmology “Cataract in the Adult Eye” Preferred Practice Patterns Guidelines recommend that topical 5% povidone-iodine drops be instilled into the conjunctival cul de sac preoperatively, whereas the Royal College of Ophthalmology Cataract Surgery Guidelines recommend a flush irrigation of 5% povidone-iodine into the conjunctival sac [23,24].

However, there are several evidences which showed that Pseudomonas cepacia and Pseudomonas aeruginosa bacteria could grow as biofilms in hospital povidone-iodine solutions [25-30]. Anderson et al., have documented the prolonged survival of Pseudomonas cepacia in commercially manufactured povidone-iodine [26]. In addition, studies documented that intrinsic contamination of povidone-iodine solutions led to both infections and pseudo infections [27,28]. Because antimicrobial efficacy of PVP-I is controversial and yet is still widely used, the present study was undertaken to measure the effectiveness of 2.5, 5, and 10% PVP-I in inhibiting the growth of both Gram negative and Gram positive bacteria.

Materials and Methods: Povidone-iodine solution preparation: 10% Povidone-Iodine (Betadine) solution (#NDC-67618-150-04, PVP-I Microbicides, Purdue Products LP, Stanford, CT, USA) was purchased from Betadine PVP-I Microbicides. 10% Povidone-Iodine solution was diluted to 5% and 2.5% in sterile 1X Phosphate Buffer Saline, PBS (pH = 7.4). Solutions were stored at room temperature until use.

Bacterial strains, media, and growth conditions: The laboratory strains of bacteria tested were S. aureus GFP AH133 [31] and P. aeruginosa PAO1 GFP strains [32], which constitutively expresses green fluorescent protein from plasmids pCM11 and pMRP9-1 respectively. The strains were routinely grown in Luria-Bertani (LB) broth at 37°C with shaking (250 rpm). To maintain pCM11 in AH133, LB was supplemented with 1 µg/mL erythromycin. To maintain pMRP9-1 in PAO1, LB broth was supplemented with 300 µg/mL carbenicillin. The clinical isolates studied were S. aureus, P. aeruginosa, S. epidermidis, S. marcescens, A. baumannii, E. coli, and two strains of S. aureus which are methicillin resistant (MRSA). The efficacy of PVP-I solution was examined using LB broth medium (#113002022, MP Biomedical, Solon, OH) and LB-Agar medium (#113002222, MP Biomedical, Solon, OH, USA) as the growth medium. The clinical isolates were obtained from the Clinical lab at Texas Tech University Health Sciences Center under an approved Institutional Review Board protocol, Texas Tech University Medical center/Lubbock, Texas, USA.

Disc diffusion assay: We used the disc diffusion testing method for our experiments as previously described [33-36]. We then analyzed any remaining bacteria on the antibiotic discs using the viable count (Colony Forming Unit; CFU) assay. The disc diffusion testing method and CFU assay are well-standardized, reliable susceptibility testing techniques.
Briefly, bacteria were grown overnight in LB medium. The following day, the bacterial culture was washed in Mueller Hinton (MH) broth (#70192, Sigma-Aldrich, St. Louis, MO, USA), and the bacterial suspension was adjusted to an OD_{600} of 0.1 (which is equivalent to the 0.5 McFarland standard; ~1 x 10^{8} bacterial cell/mL) in MH broth according to the standard guidelines of the National Committee for Clinical Laboratory Standards [33-36]. Following this, a sterile cotton swab was dipped into the adjusted bacterial culture, and a lawn of bacteria was made on a LB Agar plate using the dipped swab. The PVP-I discs were prepared by adding 20 µl of 2.5, 5, or 10% PVP-I solution onto 6 mm diameter blank BD BBL Sensi-Disc Antimicrobial Susceptibility Test Discs (#B31039, Fisher Scientific, Waltham, MA, USA). Three PVP-I discs were distributed evenly onto the LB Agar surface. Three separate plates were measured, thus nine points were determined for each bacteria. The plates were then incubated at 37 ºC for 24 h before the results were read and recorded. The diameters of the zones of complete and clear inhibition, including the diameter of the disc, were measured to the nearest millimeter with a ruler; however, the diameter of the disc was subtracted from all the measurements in the calculation and graphing.

**Confocal laser scanning microscopy (CLSM) of the GFP containing bacteria:** In addition to the ZOI study above, the discs from the S. aureus GFP AH133/pCM11 and P. aeruginosa GFP PAO1/pMPRP9-1 plates were examined under the CLSM. S. aureus GFP and Pseudomonas aeruginosa GFP are lab strains which constitutively express green fluorescent protein from plasmids pCM11 or pMPRP9-1 when grown in the presence of 1 µg/mL erythromycin or 300 µg/mL carbenicillin respectively. Images were captured using the CLSM and comparison was made between the different concentrations of PVP-I and the control. Visualization of the S. aureus GFP AH133 and P. aeruginosa PAO1 GFP bacteria was accomplished with a Nikon Eclipse Ti upright confocal laser scanning microscope (Nikon, Melville, NY, USA). Samples were examined under 10X objective lens, FITC fluorescence to eyes laser (488.0 nm), 4 channel confocal, and 512 scan size. The images were processed and analyzed using NIS-Elements AR Imaging Software.

**Determination of viable count assay:** The microorganisms remaining on the discs, following the zone of inhibition assay, of all strains were quantified by the CFU assay as previously described [37]. Following incubation, each disc piece was transferred to a sterile 1.5-mL micro centrifuge tube containing 1 mL of PBS (pH = 7.4) for enumeration of bacteria. The tubes were placed in a water bath sonicator for 10 min to loosen the cells within the disc and then vigorously vortexed 3 times for 1 min to detach the cells. Suspended cells were serially diluted (10-fold) in PBS, and 10-µL aliquots of each dilution were spotted onto LB Agar plates. In addition, the remaining 900 µL zero dilution sample was also plated on a different LB Agar plate. Thus, the equation used to calculate the count was the recovered number of colonies x dilution factor/inoculum size in mL. This means that if only one bacterial cell was originally in the tube we would have a 90% chance of detecting it. All experiments were done in triplicate on each plate, and all measurements were repeated on three separate plates. Thus, nine measurements total.

**Statistical Analyses:** Results of the viable count assays were statistically analyzed using GraphPad InStat 3.06 (GraphPad Software, San Diego, CA, USA). Significance between pairs of values (Control versus one treatment group) was calculated using an unpaired two-tailed t test when SD was not significantly different and when a Gaussian distribution was observed. If SD was significantly different, the Welch correction was applied to the unpaired two-tailed t test. When non-Gaussian distribution was observed (Kolmogorov–Smirnov test), significance was calculated by a non-parametric Mann–Whitney test. Two treatment groups were compared using the Kruskal–Wallis test (non-parametric ANOVA) with Tukey’s multiple comparisons test for significance. Differences were considered significant when the P-value was <0.05.

**Results:** As seen in Figures 1A and 2A, at a concentration of 2.5% PVP-I, most bacteria showed a ZOI greater than 6 mm (cellulose disc diameter – represented by the line drawn on Figures 1A and 2A) except P. aeruginosa PAO1 GFP strain which did not show a ZOI. At a concentration of 5% and 10% PVP-I, all bacteria tested showed ZOI. The three bacteria with smaller ZOI even at 10% PVP-I were S. epidermidis CI, P. aeruginosa CI, and P. aeruginosa PAO1 GFP.
The results of the viable count study (Figures 1B and 2B) showed different results from those obtained from the ZOI studies. The 2.5% PVP-I did not completely kill any of the tested bacteria with the S. epidermidis and P. aeruginosa strains showing no difference in number of colonies from the control trial. At 5% PVP-I, again, no strains showed complete killing except for Serratia marcescens. At 5% PVP-I, S. epidermidis showed no difference from the control. At 10% PVP-I only the S. aureus strains, including the MRSA strains, and the S. marcescens, showed complete killing of the bacteria. However, S. epidermidis, E. coli, A. baumannii strains, and both strains of P. aeruginosa, only showed incomplete killing.

Figure 1: The ZOI (A) of Gram Positive Bacteria on the Discs were Measured in mm (the Diameter of the Disc was Subtracted from Total Diameter of the Zone) and the Bacteria Remaining on the Disc was Quantified by the Viable Count Assay (B)

Figure 2: The ZOI (A) of Gram Negative Bacteria on the discs were Measured in mm (the Diameter of the Disc was Subtracted from Total Diameter of the Zone) and the Bacteria Remaining on the Disc was Quantified by the Viable Count Assay (B)

The confocal laser scanning microscopy results confirmed the viable count results for S. aureus GFP AH133 and P. aeruginosa PAO1 GFP. As shown in Figure 3A-D, only 10% PVP-I completely killed S. aureus GFP AH133 whereas there were still a lot of bacteria present on the discs treated with 2.5% and 5% PVP-I. In addition, 10% PVP-I just partially killed P. aeruginosa PAO1 GFP as shown in Figure 3E-H. The color indicates that the bacteria are alive. The color is lost when the bacteria dies. The results for the size of the zones of clearance around the PVP-I discs for all microorganisms on Agar plates are shown in Table 1.
Figure 3: Confocal Laser Scanning Microscopy Images of the *S. aureus* GFP AH133 (A,B,C,D), and the *P. aeruginosa* PAO1 GFP (E,F,G,H), that Remained on the PVP-I Discs. Bar Scale Equals to 100 µm

Table 1: Comparison of the Mean Size of the Inhibition Zones Around the PVP-I Discs

Discussion: An important conclusion from our experiments is the observation that the disc diffusion assay does not accurately assess the ability of an antimicrobial to kill bacteria. This is based upon the finding that there were several instances of large zones of inhibition that subsequently produced numerous bacteria in our viable count assay. This is best evidenced by the 5% concentration of PVP-I, where every strain of bacteria demonstrated an increase in its ZOI from that shown by the 2.5% concentration. However, there was not a consistent change on the viable count assay. In some cases, there was no change and in some cases there was a change of up to 6 logs. This is important because the disc diffusion assay has long been used to determine the effectiveness of antimicrobials at killing different strains of bacteria. Similar results to the viable count results were seen from the images by the use of confocal laser scanning microscopy in Figure 3. In this case large amounts of bacteria were visualized for both Gram-negative and Gram-positive bacteria in 5% PVP-I. Also, in the case of *P. aeruginosa*, significant amounts of bacteria are seen in 10% PVP-I.

It is important to note the way the assays were carried out for the disc diffusion assay. For this study, the PVP-I was added to a disk that was immediately placed upon an Agar plate upon which had just been spread a lawn of bacteria. At the end of a 24 hour time period, the size of the zone of inhibition was measured including the diameter of the disc (6 mm);
however, the diameter if the disc was minus from the calculations. This would appear to measure the ability of the PVP-I to kill the bacteria (bactericidal activity). These same discs were then used to measure the amount of bacteria in the disc by the viable count assay. For many of the bacteria tested, there were significant zones of inhibitions, and yet there were live bacteria found in the disc (10^3 to 10^5). Since the original bacterial lawn spread on the whole 100 mm diameter Agar plate only contained around 10^5 bacterial cells, this would indicate that significant growth happened in the disc in the presence of PVP-I. The appearance of a zone of inhibition was due to a difference in growth rate and not to significant killing. This finding is seen in Figures 1 and 2, where significant zones of inhibition are seen in many cases with 5% PVP-I and yet 1 x 10^6 or higher numbers of bacteria are found on many of the discs.

These results are consistent with those found in a patient study on the use of PVP-I after intravitreal injection [38]. In this study, 5% PVP-I was placed on the eye for different time periods. Their results showed roughly a 40-50% reduction in the number of positive cultures after treatment. While this was listed as a significant reduction, it still means that in many cases bacteria survive the treatment. This clinical study was also limited by the fact that not all bacteria can be cultured so there could be many more bacteria than those measured. Thus, while PVP-I is widely used after procedures in the eye, it is limited in its killing ability.

It is also evident that bacteria do not have to form a biofilm to be resistant to PVP-I. This is the case because since the PVP-I was added to the disc prior to its addition to the dish containing the bacteria, the bacteria would not have had a chance to form a biofilm to be resistant to the PVP-I. Apparently all PVP-I does in most cases is slow down the growth of the bacteria, but not kill it. Thus one can see why contamination by different bacteria can occur in a PVP-I bottle.

In conclusion, the zone of inhibition assay does not give a realistic assessment of the ability of an antimicrobial to kill bacteria. In addition, both 2.5 and 5% PVP-I are not effective at killing all the bacteria tested (with the exception of S. marcescens), and even 10% PVP-I is not effective against P. aeruginosa, S. epidermidis, E. coli, and A. baumannii strains invitro. It is important to note that 5% PVP-I, which is the most commonly used concentration, was only effective at killing one bacteria (S. marcescens) out of the 10 different bacterial strains tested. Thus, PVP-I is not very efficacious as a means of killing bacteria after a surgical procedure.

References:


