

## NATIONAL MASTITIS COUNCIL

### RECOMMENDED PROTOCOLS FOR EVALUATING EFFICACY OF POSTMILKING TEAT GERMICIDES

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#### Introduction

The role of postmilking teat dipping as a management tool in preventing new intramammary infections (IMI) in dairy cows is well documented (Dodd et al., 1969; Farnsworth, 1980; Natzke, 1977; Pankey et al., 1984; Philpot, 1979). Success of this practice in reducing new IMI has resulted in the emergence of numerous germicidal products marketed as teat dips. However, governmental agency requirements for data on the ability of a teat dip to reduce incidence of new IMI (efficacy data) are inconsistent among countries. One objective of the National Mastitis Council (NMC) is to monitor the development of protocols that determine effectiveness of mastitis control products, such as teat disinfectant solutions. The NMC Research Committee and Teat Dip Committee collaborated to review and update guidelines and protocols to standardize procedures for the evaluation of postmilking teat dips and to assure uniform and accurate comparison of studies that evaluate these products.

In 2003, the NMC Board of Directors voted to amend the recommended protocols for teat dip efficacy testing. Revisions were made to update technologies, enhance scientific merit, improve statistical analyses, and further assure standardization of testing procedures. Major revisions to previously published protocols (Pankey et al., 1983; Hogan et al., 1990) include 1) standardization of challenge organisms for experimental exposure trials; 2) updating the procedures for statistical analyses; 3) establishing appropriate sample size to detect a difference between treatments; 4) standardization of milking systems operations and udder preparation procedures; and 5) removal of the requirements for at least two herds and a trial length of at least 12 months from the natural exposure protocol.

The four postmilking teat dip protocols are designed to 1) determine efficacy of a teat dip in preventing new IMI following experimental exposure of teats to mastitis pathogens; 2) determine efficacy of a teat dip in preventing naturally occurring IMI; 3) compare the efficacy of an experimental teat dip with that of a positive control product in reducing naturally occurring IMI; and 4) determine efficacy of a barrier teat dip based on reduction of naturally occurring new IMI. Design, procedures, and guidelines for data analysis for each protocol are described.

## **Determining the Efficacy of a Postmilking Teat Dip Following Experimental Exposure of Teats to Mastitis Pathogens**

Experimental Design. All four teats of each cow are exposed to suspensions of *Staphylococcus aureus*, *Streptococcus agalactiae*, and/or other mastitis pathogens on a repeated experimental basis. Teat dip is applied to two teats of each udder, remaining teats serve as undipped controls, and the numbers of new intramammary infections (IMI) in quarters with dipped and control teats are determined.

Selection of Experimental Herds, Cows, and Quarters. Trials should be conducted in university research herds or herds supervised by university research faculty. Milking equipment and milking management procedures should be monitored carefully and regularly as described below to minimize machine-mediated infections. All quarters are eligible for inclusion in the trial except those infected with microorganisms of the same species as challenge microorganisms and quarters in which teats are deformed, abnormal, or injured prior to or during the trial. Two diagonal teats or teats on either the right or left side of each udder are immersed in the experimental product while remaining teats serve as controls; comparisons between front and rear quarters should not be made.

Cultures. Trials should be conducted with reference strains of bacteria. *Staph. aureus* American Type Culture Collection (ATCC 29740; Rockville, MD) and *Strep. agalactiae* ATCC 27956 are highly recommended. The Hill Farm Research Station at Louisiana State University, Homer, LA (LSU) serves as the primary repository for isolates of *Staph. aureus* (ATCC 29740) and *Strep. agalactiae* (ATCC 27956). The Experiment Station at Washington State University, Pullman, WA (WSU) serves as the secondary (back-up) repository for these isolates. All isolates are stored at -70° C in tryptic soy broth/glycerol media, 80:20 ratio. Stock cultures of the isolates are to be obtained prior to each study from LSU or WSU for a charge of \$50.00, and cultures will be shipped via overnight express service in suitable transport media.

A sufficient number of vials of stock culture should be prepared and stored, with minimal serial transfer, to permit preparation of an actively growing culture from a separate vial each time bacteria are prepared for challenge throughout the trial. Although test organisms recommended above are not known to be pathogenic to humans, it is prudent to use care in handling all bacterial cultures. Unused challenge suspensions should be autoclaved before disposal, and personnel should be instructed to sanitize hands with an antiseptic soap after contact with any challenge suspensions or milk from contaminated cows.

Preparation of *Staph. aureus*. One 6-ml tube of Trypticase Soy Broth (TSB; BBL Microbiology Systems, Cockeysville, MD) is inoculated from a vial of stored stock culture or from 2 to 3 colonies of plated stock culture, and incubated at 37°C for 5 to 7 h. The culture is then streaked onto a Trypticase Soy Agar (BBL Microbiology Systems) plate containing 5% calf or sheep blood as a purity check. The suitability of blood for this purpose should be determined as described previously (Harmon et al., 1990). At the same time as blood agar plates are plated and cultured for a purity check, a 500-ml flask of TSB is inoculated with the entire contents of the 6-ml culture and incubated for 16 to 18 h at 37°C on a gyratory shaker. Bacterial cells are pelleted by centrifugation (3,000-4,000 x g for 15 min), washed twice with 0.1% proteose-peptone No.3 (Difco Laboratories, Detroit, MI), and resuspended in 50 ml of 0.1% proteose-peptone. A

standard plate count is conducted on the stock suspension and then stored at 5 to 7°C. This plate count is used to determine the dilution required to prepare an initial challenge suspension containing  $\approx 5 \times 10^7$  cfu/ml in TSB or skim milk broth media. The challenge suspension is prepared immediately before use when practical. The stock suspension may be maintained in proteose-peptone at 5°C for up to 1 wk (depending upon viability) for use in the preparation of daily challenge suspensions. In all cases, the suspension should be mixed thoroughly before dispensing, and a standard plate count on each daily challenge suspension should be conducted. Alternative preparation techniques that repeatedly produce challenge suspensions of the desired bacterial concentration and result in the desired rate of IMI following experimental exposure of teats may be used.

Preparation of *Strep. agalactiae*. Cultures of *Strep. agalactiae* are prepared each day of challenge by inoculating four 6-ml vials of TSB with stored stock culture or from 2 to 3 colonies of plated stock culture. The 6-ml culture tubes are incubated for 15 h at 37°C, and then the entire contents of four tubes are inoculated into a 500-ml flask of TSB, which is incubated for 6 to 7 h at 37°C on a gyratory shaker. The culture is diluted using TSB or skim milk broth media to produce a culture concentration of  $\approx 5 \times 10^7$  cfu/ml as determined by optical density using a spectrophotometer (620 nm). Alternative preparation techniques that repeatedly produce challenge suspensions of the desired bacterial concentration and result in the desired rate of IMI following experimental exposure of teats may be used.

When both *Staph. aureus* and *Strep. agalactiae* are used for the experimental challenge, as is usually the case, a portion of the *Staph. aureus* stock suspension is incorporated into the *Strep. agalactiae* TSB suspension and diluted with skim milk broth media to achieve  $\approx 5 \times 10^7$  cfu/ml of each pathogen.

Milking System Evaluation and Premilking Udder Preparation. A milking system evaluation should be performed prior to trial initiation to assure that all machinery meets the requirements set forth by the NMC Procedures for Evaluating Vacuum Levels and Air Flow in Milking Systems. Premilking udder preparation methods should be decided before the start of the trial and recorded for inclusion in the final report. For studies requiring no premilking disinfection, premilking udder preparation should consist of the use of single service water-moistened towels (free of sanitizer, one towel per cow) to wet and clean the teats prior to fore-stripping. Fore-stripping is accomplished by expressing two squirts of milk. For studies requiring that a premilking teat disinfectant be used, each quarter should be fore-stripped by expressing two squirts of milk followed by dipping of all teats with the designated premilking disinfectant. A 30-s contact time should be allowed and the teats wiped dry using a single service towel before attaching the milking unit. The premilking disinfectant used should have been previously demonstrated to be effective through NMC recommended testing and listed in the NMC Teat Dip Bibliography.

Experimental Exposure of Teats and Teat Disinfection. Each teat is challenged by immersion to a depth of approximately 25 mm in a beaker or other suitable vessel of a freshly prepared TSB suspension of the test organism(s). Teats should be challenged immediately after removal of the milking machine. Fresh TSB suspension of test microbe(s) should be added as needed to maintain a depth of approximately 25 mm of challenge suspension in each vessel. Challenge

should be performed at least once daily for a minimum of 5 d each wk. Teat dip should be applied to the distal 25 mm of teats immediately after exposure to the challenge suspension.

Sampling Schedules and Procedures. Duplicate milk samples are collected from each lactating quarter to determine bacteriological status of quarters within 7 d prior to initiation of the trial. A third sample is collected when results of the first two samples differ. Quarter foremilk samples are collected weekly during the trial. If any quarter secretes abnormal milk or develops signs of clinical mastitis, duplicate samples should be obtained before antibiotic treatment. All samples should be collected immediately prior to a regular milking. Sampling procedures are as described (Dodd et al., 1969). Briefly, three or four streams of foremilk are discarded from each quarter before sanitizing teat ends with cotton swabs and collecting samples. Sterile swabs should be soaked in 70% alcohol from which excess fluid has been expressed; no more than two teats should be sanitized per swab. The far teats should be sanitized first and the near teats last to avoid contaminating the near teats with the sampler's fingers. When collecting milk samples, the near teats should be sampled first and the far teats last to avoid contaminating the near teats with the sampler's fingers. This sanitization and collection procedure reduces the number of the bacteriologically false-positive samples that may result from the large numbers of experimentally applied bacteria, which may be present at the teat orifice.

Criteria for Diagnosing Infections. All milk samples should be examined microbiologically according to standard procedures (Harmon et al., 1990). A new IMI in a quarter is diagnosed when the same bacterial species is isolated from 1) two consecutive samples during the trial ( $\geq 500$  cfu/ml); 2) a single sample from a quarter with clinical mastitis ( $\geq 100$  cfu/ml); or 3) three consecutive samples during the trial ( $\geq 100$  cfu/ml). An individual quarter is eligible for only one new IMI per bacterial species during the trial. A quarter may receive treatment after a new IMI is confirmed, but the quarter is not eligible for another new IMI by the same pathogen.

Statistical Analysis. Data on efficacy of a teat dip formulation are examined in terms of mean percentage reduction in rate of new IMI achieved among dipped quarters compared with the rate among control quarters, and the statistical reliability of the mean percentage reduction. Data must express the relation between quarters becoming infected in quarters treated with the test dip and in undipped control quarters. Differences between the percentage of quarters becoming infected between treatments can be tested with the standard normal approximation (Steel and Torrie, 1980). For a given time period, this difference is determined as follows:

Let:

x1 = number new IMI in control quarters,  
x2 = number new IMI in treated quarters,  
n1 = number of eligible control quarters, and  
n2 = number of eligible treated quarters.

Then calculate:

p1 (fraction of new IMI in control quarters) =  $x1/n1$ ,  
p2 (fraction of new IMI in treated quarters) =  $x2/n2$ ,  
p (pooled infection proportion) =  $(x1+x2) / (n1+n2)$ ,  
P (% reduction in new IMI) =  $1 - p2/p1$ , and  
Z =  $[ x1/n1 - x2/n2 ] / \text{sqrt}[ p*(1-p)/n1 + p*(1-p)/n2 ]$ .

The statistical significance of the test can then be evaluated from one-tailed standard normal tables using the Z statistic (Steele and Torrie, 1980).

An example based on actual new IMI data in an experimental infection trial (Boddie and Nickerson, 1997) is given below using the Z formula described above:

x1 = number new IMI in control quarters = 23  
 x2 = number new IMI in treated quarters = 13  
 n1 = number of eligible control quarters = 161  
 n2 = number of eligible treated quarters = 154

Calculate:

p1 (% new IMI in control quarters) =  $x1/n1 = 23/161 = 0.143$   
 p2 (% new IMI in treated quarters) =  $x2/n2 = 13/154 = 0.084$   
 p (pooled infection proportion) =  $(x1+x2)/(n1+n2) = (23+13)/(161+154) = 36/315 = 0.114$   
 P (% reduction in new IMI) =  $1 - p2/p1 = 1 - 0.084/0.143 = 41.26\%$   
 $Z = [(23/161)-(13/154)]/\sqrt{0.114(1-0.114)/161 + 0.114(1-0.114)/154} = 1.63888$

Thus, the percentage reduction in new IMI is 41.26%, and from one-tailed standard normal probability tables, a Z value of 1.63 approaches significance at the  $P \leq 0.05$  level, demonstrating no difference in percentage new IMI between control (14.3%) and treated (8.4%) quarters.

Sample size (n) to detect a difference between two treatment groups. Sample size per treatment group can be calculated by the following steps proposed by Casagrande et al. (1978).

1. Determine the absolute value for a one-tailed test, and search the cumulative standard normal distribution function tables for:

Z1 = critical one-tailed value for Type I error rate (usually 5%): Locate the 0.95 probability and the corresponding  $Z_i$  value, which by interpolation is 1.645.

Z2 = critical one-tailed value for Type II error rate (usually 10%): Locate the 0.90 probability and the corresponding  $Z_i$  value, which by interpolation is 1.281.

2.  $p$  (average infection rate) =  $[p1$  (control infection rate) +  $p2$  (experimental infection rate)]/2.
3.  $A = [ Z1*\sqrt{2*p*(1-p)} + Z2*\sqrt{p1*(1-p1) + p2*(1-p2)} ]^2$  .
4. D (difference anticipated between two groups) =  $p1$  (control infection rate) -  $p2$  (experimental infection rate).
5.  $n = A*[1 + \sqrt{1 + 4D/A}]^2 / (4*D*D)$ ,  
 which will be the number of eligible quarters needed per treatment group.

For example (see Table 1 below), if quarters in the control group (p1) are expected to have a 20% infection rate (0.20), and quarters in the experimental group (p2) are expected to have a 5% infection rate (0.05), and average infection rate (p) is 0.125, and the difference (D) between infection rates is 0.15, and it is desirable to have a 90% chance of detecting a statistical difference (Type II error rate) at the 5% significance level (Type I error rate), then A is determined as follows:

$$A = [ 1.645 * \sqrt{2 * 0.125 * (1 - 0.125)} + 1.281 * \sqrt{0.20 * (1 - 0.20) + 0.05 * (1 - 0.05)} ]^2$$

$$A = 1.830347$$

Then determine  $n = (A * [1 + \sqrt{1 + 4D/A}]^2) / (4 * D * D)$

$$n = (1.830347 * [1 + \sqrt{1 + 4(0.15)/1.830347}]^2) / (4 * 0.15 * 0.15) = 95$$

Table 1 contains sample sizes for various infection rates.

Table 1. Number of eligible quarters per treatment group needed in comparing two proportions with a one-tailed test, assuming 90% power and 5% significance level.

Control infection rate (%)	Treatment infection rate (%)			
	5	10	15	20
30	45	76	144	339
25	62	121	292	1232
20	95	236	1027	---
15	172	787	---	---
10	513	---	---	---

The length of a trial to demonstrate acceptable efficacy of a teat dip depends on 1) number of quarters tested; 2) rate of new IMI in dipped quarters; and 3) percentage reduction of IMI in dipped quarters. The greater the amount by which the mean reduction exceeds the minimum acceptable, the smaller the size of trial needed to determine efficacy. For groups of a given size, the number of quarters required to become infected in the control group varies depending on the extent to which the dip is successful in reducing the incidence of infection in the dipped group.

Presentation of Data. The report of a trial should include 1) duration of the trial; 2) number of quarters initially eligible for infection; 3) number of new IMI by each test organism in control and treated quarters; 4) percentage reduction in new IMI by test organism(s) in treated quarters; and 5) the cfu/ml of bacteria in each challenge suspension. Teats may be examined weekly throughout the trial and any abnormalities of individual teats recorded. Scaling, chapping, pox lesions, and teat erosion around the teat orifice should be noted. In addition, teat condition when clinical mastitis occurs should be recorded.

## **Determining the Efficacy of a Postmilking Teat Dip in Preventing New Naturally Occurring Intramammary Infections**

Experimental Design. Teats of half of the quarters are immersed after each milking in the teat dip being tested; remaining teats serve as undipped controls. The experimental design can be either a split-herd or a split-udder design. The numbers of new intramammary infections (IMI) that occur in quarters with teats dipped in the experimental product and the number of new IMI in control quarters with undipped teats are determined.

Selection of Experimental Herds, Cows, and Quarters. Trials should be conducted in herds in which wholehearted cooperation of managers can be attained to comply with experimental procedures. Milking equipment and milking management practices should be monitored carefully and regularly as described below to minimize machine-mediated infections. This is especially necessary in commercial herds in which constant supervision by the investigator will not be practical. All quarters are eligible for new IMI except quarters with teats that are deformed due to previous injury. Quarters with teats that are injured during the trial should be excluded for the remainder of that lactation; such quarters may re-enter the trial after a dry period if the injury has healed.

Milking System and Premilking Udder Preparation. A milking system evaluation should be performed prior to trial initiation to assure that all machinery meets the requirements set forth by the NMC Guidelines for Milking System Evaluations. Premilking udder preparation methods should be decided before the start of the trial and recorded for the final report. For studies requiring no premilking disinfection, premilking udder preparation should consist of the use of single service water-moistened towels (free of sanitizer, one per cow) to wet and clean the teats prior to fore-stripping. Fore-stripping is accomplished by expressing two squirts of milk. For studies requiring that a premilking disinfectant be used, each quarter should be fore-stripped by expressing two squirts of milk followed by dipping of all teats with the designated premilking disinfectant. A 30-s contact time should be allowed and the teats wiped dry using a single service towel before attaching the milking unit. The premilking disinfectant used should have been previously demonstrated to be effective through NMC recommended testing and listed in the NMC Teat Dip Bibliography.

Teat Dipping. Teats of half of the cows are dipped in the experimental product in a split-herd design, and cows in the remainder of the herd serve as undipped controls. In this design, cows should be balanced by parity, stage of lactation, and bacteriological status of quarters. The two groups of cows should be milked by the same personnel in the same facility. If two different housing conditions exist, the two treatment groups should be equally exposed to the two housing environments. Each treatment group should be identified with leg bands and/or neck chains.

When a half-udder design is used, either two diagonal teats or teats on either the right or left side of each udder are dipped with the experimental product. Teat dip is applied immediately after milking machine removal.

Sampling Schedule and Procedures. Duplicate quarter milk samples are collected to determine existing infections in the herd within 7 d prior to the beginning of the trial. A third sample is obtained when results of the first two samples differ. Single quarter milk samples are collected

and cultured monthly or duplicate samples are collected and cultured bimonthly during the trial. Duplicate samples from cows collected 3 d post calving and herd additions should be cultured prior to inclusion in the trial. When any quarter develops clinical mastitis, duplicate milk samples from all four quarters should be cultured before any treatment is administered. In addition, duplicate milk samples should be collected and cultured from individual cows at drying off, leaving the study, or upon leaving the herd. All milk samples should be collected immediately before a regular milking using standard procedures (Harmon et al., 1990). Briefly, three or four streams of foremilk are discarded from each quarter before sanitizing teat ends with cotton swabs and collecting samples. Sterile swabs should be soaked in 70% alcohol from which excess fluid has been expressed; no more than two teats should be sanitized per swab. The far teats should be sanitized first and the near teats last to avoid contaminating the near teats with the sampler's fingers. When collecting milk samples, the near teats should be sampled first and the far teats last to avoid contaminating the near teats with the sampler's fingers.

Criteria for Diagnosing Infections. All milk cultures are examined microbiologically and the microorganisms isolated are identified according to standard procedures (Harmon et al., 1990). In determining that a quarter is free of infection when it enters the trial, no pathogens may be recovered from two of the initial duplicate or consecutive samples. An IMI is diagnosed when the same bacterial species is isolated from both of the duplicate samples taken bimonthly or from clinical quarters, or from two consecutive monthly samples collected during the trial. The status of a quarter exhibiting clinical mastitis should be recorded as a bacteriologically-negative clinical case when bacteriological results of duplicate samples from a clinical quarter do not match. Clinical mastitis and IMI diagnosed during the first 7 d of lactation should not be included in data analyses of teat dip efficacy.

An individual quarter is eligible for only one infection per bacterial species during a lactation (i.e., only one *Escherichia coli* infection per quarter per lactation). Quarters infected in one lactation may be included in the trial in the subsequent lactation if it is determined that the infection was eliminated during the dry period either spontaneously or as a result of nonlactating cow therapy.

Statistical Analyses. Data on efficacy of a teat dip formulation are examined in terms of mean percentage reduction in rate of new IMI achieved among dipped quarters compared with the rate among control quarters, and the statistical reliability of the mean percentage reduction. Data must express the relation between quarters becoming infected in quarters treated with the test dip and in undipped control quarters.

Differences between the percentage of quarters becoming infected between treatments can be tested with the standard normal approximation (Steel and Torrie, 1980). For a given time period, this difference is determined as follows:

Let:

- x1 = number new IMI in control quarters,
- x2 = number new IMI in treated quarters,
- n1 = number of eligible control quarters, and
- n2 = number of eligible treated quarters.

Then calculate:

$$\begin{aligned} p_1 \text{ (fraction of new IMI in control quarters)} &= x_1/n_1, \\ p_2 \text{ (fraction of new IMI in treated quarters)} &= x_2/n_2, \\ p \text{ (pooled infection proportion)} &= (x_1+x_2) / (n_1+n_2), \\ P \text{ (% reduction in new IMI)} &= 1 - p_2/p_1, \text{ and} \\ Z &= [ x_1/n_1 - x_2/n_2 ] / \text{sqrt}[ p*(1-p)/n_1 + p*(1-p)/n_2 ]. \end{aligned}$$

The statistical significance of the test can then be evaluated from one-tailed standard normal tables using the Z statistic (Steele and Torrie, 1980).

An example based on total new *Staph. aureus* IMI in dipped and control quarters in an efficacy trial (Nickerson et al., 1986) using a 0.5% iodophor is given below using the formula described above:

$$\begin{aligned} x_1 &= \text{number new IMI in control quarters} = 43 \\ x_2 &= \text{number new IMI in treated quarters} = 14 \\ n_1 &= \text{number of eligible control quarters} = 309 \\ n_2 &= \text{number of eligible treated quarters} = 320 \end{aligned}$$

Calculate:

$$\begin{aligned} p_1 \text{ (fraction of new IMI in control quarters)} &= x_1/n_1 = 43/309 = 0.139 \\ p_2 \text{ (fraction of new IMI in treated quarters)} &= x_2/n_2 = 14/320 = 0.044 \\ p \text{ (pooled infection proportion)} &= (x_1+x_2)/(n_1+n_2) = (43+14)/(309+320) = 57/629 = 0.091 \\ P \text{ (% reduction in new IMI)} &= 1 - p_2/p_1 = 1 - 0.044/0.139 = 68.35\% \\ Z &= [(43/309)-(14/320)]/\text{sqrt}[ 0.091(1-0.091)/309 + 0.091(1-0.091)/320 ] = 4.167 \end{aligned}$$

Thus, the percentage reduction in new IMI is 68.35%, and from one-tailed standard normal probability tables, a Z value of 4.167 is significant at the  $P \leq 0.0001$  level, demonstrating a difference in percentage of new IMI between control (13.9%) and treated (4.4%) quarters.

Sample size (n) to detect a difference between two treatment groups. Sample size per treatment group can be calculated by the following steps proposed by Casagrande et al. (1978).

1. Determine the absolute value for a one-tailed test, and search the cumulative standard normal distribution function tables for:

$Z_1$  = critical one-tailed value for Type I error rate (usually 5%): Locate the 0.95 probability and the corresponding  $Z_1$  value, which by interpolation is 1.645.

$Z_2$  = critical one-tailed value for Type II error rate (usually 10%): Locate the 0.90 probability and the corresponding  $Z_1$  value, which by interpolation is 1.281.

2.  $p$  (average infection rate) =  $[p_1$  (control infection rate) +  $p_2$  (experimental infection rate)]/2.

3.  $A = [ Z_1*\text{sqrt}(2*p*(1-p)) + Z_2*\text{sqrt}(p_1*(1-p_1) + p_2*(1-p_2)) ]^2$ .

4.  $D$  (difference anticipated between two groups) =  $p_1$  (control infection rate) -  $p_2$  (experimental infection rate).
5.  $n = A * [1 + \sqrt{1 + 4D/A}]^2 / (4 * D * D)$ ,  
which will be the number of eligible quarters needed per treatment group.

For example (see Table 1 below), if quarters in the control group ( $p_1$ ) are expected to have a 15% infection rate (0.15), and quarters in the experimental group ( $p_2$ ) are expected to have a 5% infection rate (0.05), and average infection rate ( $p$ ) is 0.10, and the difference ( $D$ ) between infection rates is 0.10, and it is desirable to have a 90% chance of detecting a statistical difference (Type II error rate) at the 5% significance level (Type I error rate), then  $A$  is determined as follows:

$$A = [ 1.645 * \sqrt{2 * 0.10 * (1 - 0.10)} + 1.281 * \sqrt{0.15 * (1 - 0.15) + 0.05 * (1 - 0.05)} ]^2$$

$$A = 1.522665$$

Then determine  $n = (A * [1 + \sqrt{1 + 4D/A}]^2 / (4 * D * D))$   
 $n = (1.522665 * [1 + \sqrt{1 + 4(0.10) / 1.522665}]^2 / (4 * 0.1 * 0.1)) = 172$

Table 1 contains sample sizes for various infection rates.

Table 1. Number of eligible quarters per treatment group needed in comparing two proportions with a one-tailed test, assuming 90% power and 5% significance level.

Control infection rate (%)	Treatment infection rate (%)			
	5	10	15	20
30	45	76	144	339
25	62	121	292	1232
20	95	236	1027	---
15	172	787	---	---
10	513	---	---	---

The length of a trial to demonstrate acceptable efficacy of a teat dip depends on 1) number of quarters tested; 2) rate of new IMI in dipped quarters; and 3) percentage reduction of IMI in dipped quarters. The greater the amount by which the mean reduction exceeds the minimum acceptable, the smaller the size of trial needed to determine efficacy. For groups of a given size, the number of quarters required to become infected in the control group varies depending on the extent to which the dip is successful in reducing the incidence of infection in the dipped group.

Presentation of Data. The report of a trial should include 1) duration of the trial; 2) number of quarters in the trial at the onset and on the date of each monthly or bimonthly sampling; 3) number of total new IMI, categorized by bacterial species or type, that occurred in control and treated quarters; 4) the percentage differences in total new IMI between treated and control quarters and for each bacterial species; 5) the number of new clinical cases, categorized by bacteriological status that occurred in control and treated quarters; and 6) the percentage difference in new clinical cases between treated and control quarters.

## **Comparing an Experimental Postmilking Teat Dip with a Product of Known Efficacy in Reducing Incidence of New Naturally Occurring Intramammary Infections**

Experimental Design. Teats of half of the quarters are immersed after each milking in the teat dip being tested. The experimental design can be either a split-herd or a split-udder design. The number of new intramammary infections (IMI) that occur in quarters with teats dipped in the experimental product and in positive control quarters are determined. The positive control product must be a teat dip shown to be efficacious compared with not dipping in both experimental and natural challenge trials [check this out], and must be cited in the NMC Teat Dip Bibliography.

Selection of Experimental Herds, Cows, and Quarters. Trials should be conducted in herds in which wholehearted cooperation of managers can be attained to comply with experimental procedures. Milking equipment and milking management practices should be monitored carefully and regularly as described below to minimize machine-mediated infections. This is especially necessary in commercial herds in which constant supervision by the investigator will not be practical. All quarters are eligible for new IMI except quarters with teats that are deformed due to previous injury. Quarters with teats that are injured during the trial should be excluded for the remainder of that lactation; such quarters may re-enter the trial after a dry period if the injury has healed.

Milking System and Premilking Udder Preparation. A milking system evaluation should be performed prior to trial initiation to assure that all machinery meets the requirements set forth by the NMC Procedures for Evaluating Vacuum Levels and Air Flow in Milking Systems. Premilking udder preparation methods should be decided before the start of the trial and recorded for the final report. For studies requiring no premilking disinfection, premilking udder preparation should consist of the use of single service water-moistened towels (free of sanitizer, one per cow) to wet and clean the teats prior to fore-stripping. Fore-stripping is accomplished by expressing two squirts of milk). For studies requiring that a disinfectant be used, each quarter should be fore-stripped by expressing two squirts of milk followed by dipping of all teats with the designated premilking disinfectant. A 30-s contact time should be allowed and the teats wiped dry using a single service towel before attaching the milking unit. The premilking disinfectant used should have been previously demonstrated to be effective through NMC recommended testing and listed in the NMC Teat Dip Bibliography.

Teat Dipping. All teats of half the cows are immersed in the experimental product in a split-herd design. Cows in the remainder of the herd serve as positive controls and all teats are dipped in a product of known efficacy. Cows in experimental groups should be balanced by parity, stage of lactation, and bacteriological status. The two groups should be milked by the same personnel in the same facility. If two different housing conditions exist, the two treatment groups should be equally exposed to the two housing environments. Each treatment group should be identified with leg bands and/or neck chains.

When a half-udder design is used, two teats are dipped in the experimental product and the other two teats are dipped in the positive control product. Experimental and positive control teat dips are applied immediately after milking machine removal.

Sampling Schedule and Procedures. Duplicate quarter milk samples are collected to determine existing infections in the herd within 7 d prior to the beginning of the trial. A third sample is obtained when results of the first two samples differ. Single quarter milk samples are collected and cultured monthly or duplicate samples are collected and cultured bimonthly during the trial. Duplicate samples from cows collected 3 d post calving and herd additions should be cultured prior to inclusion in the trial. When any quarter develops clinical mastitis, duplicate milk samples from all four quarters should be cultured before any treatment is administered. In addition, duplicate milk samples should be collected and cultured from individual cows at drying off, leaving the study, or upon leaving the herd. All milk samples should be collected immediately before a regular milking using standard procedures (Harmon et al., 1990). Briefly, three or four streams of foremilk are discarded from each quarter before sanitizing teat ends with cotton swabs and collecting samples. Sterile swabs should be soaked in 70% alcohol from which excess fluid has been expressed; no more than two teats should be sanitized per swab. The far teats should be sanitized first and the near teats last to avoid contaminating the near teats with the sampler's fingers. When collecting milk samples, the near teats should be sampled first and the far teats last to avoid contaminating the near teats with the sampler's fingers.

Criteria for Diagnosing Infections. All milk cultures are examined bacteriologically and microorganisms isolated are identified according to standard procedures (Harmon et al., 1990). In determining that a quarter is free of infection when it enters the trial, no pathogens may be recovered from two of the initial duplicate or consecutive samples. An IMI is diagnosed when the same bacterial species is isolated from both of the duplicate samples taken bimonthly or from clinical quarters, or from two consecutive monthly samples collected during the trial. The status of a quarter exhibiting clinical mastitis should be recorded as a bacteriologically-negative clinical case when bacteriological results of duplicate samples from a clinical quarter do not match. Clinical mastitis and IMI diagnosed during the first 7 d of lactation should not be included in data analyses of teat dip efficacy.

An individual quarter is eligible for only one infection per bacterial species during a lactation (i.e., only one *Escherichia coli* infection per quarter per lactation). Quarters infected in one lactation may be included in the trial in the subsequent lactation if it is determined that the infection was eliminated during the dry period either spontaneously or as a result of nonlactating cow therapy.

The use of a positive control is most advantageous when it is impractical to not dip a proportion of teats in the herd. The purpose of the trial must be decided *a priori* when using a positive control, i.e., if the experimental product is more efficacious than the positive control or if the efficacy of the experimental product does not vary from that of the positive control by greater than a predetermined amount. To determine if the efficacy of an experimental product is greater than that of a positive control, the hypothesis is formulated and tested as if teats of positive control quarters were not being dipped. Experimental products may also be tested to determine if efficacy is equal to that of the positive control. Equivalence can be evaluated by constructing a confidence interval on the difference between two proportions in the statistical analysis below.

Statistical Analyses. A 95% one-sided lower limit (LLCI) confidence interval for the difference between proportions can be computed using the normal approximation and the critical one-tailed Z of 1.645. For a given time period, this difference is determined as follows:

Let:

$x_1$  = number new IMI in control quarters,  
 $x_2$  = number new IMI in treated quarters ,  
 $n_1$  = number of eligible control quarters, and  
 $n_2$  = number of eligible treated quarters.

Then calculate:

$p_1$  (fraction of new IMI in control quarters) =  $x_1/n_1$ ,  
 $p_2$  (fraction of new IMI in treated quarters) =  $x_2/n_2$ ,  
 $p$  (pooled infection proportion) =  $(x_1+x_2) / (n_1+n_2)$ ,  
 $P$  (% reduction in new IMI) =  $1 - p_2/p_1$ , and  
 $Z = [ x_1/n_1 - x_2/n_2 ] / \text{sqrt}[ p*(1-p)/n_1 + p*(1-p)/n_2 ]$ .

$$LLCI = [ p_1 - p_2 ] - 1.645 * \text{sqrt}[ p*(1-p)/n_1 + p*(1-p)/n_2 ]$$

If this one-tailed lower confidence limit is greater than or equal to zero, then the experimental product is at least as efficacious as the control.

An example (Pankey et al., 1985) based on total new *Staph. aureus* IMI in quarters dipped in an experimental formulation compared with a positive control is given below:

$x_1$  = number new IMI in positive control quarters = 31  
 $x_2$  = number new IMI in experimental product quarters = 22  
 $n_1$  = number of eligible positive control quarters = 83  
 $n_2$  = number of eligible experimental product quarters = 100

Calculate:

$p_1$  (fraction of new IMI in positive control quarters) =  $x_1/n_1 = 31/83 = 0.373$   
 $p_2$  (fraction of new IMI in experimental product quarters) =  $x_2/n_2 = 22/100 = 0.220$   
 $p$  (pooled infection proportion) =  $(x_1+x_2)/(n_1+n_2) = (31+22)/(83+100) = 53/183 = 0.289$   
 $P$  (% reduction in new IMI) =  $1 - p_2/p_1 = 1 - 0.220/0.373 = 41.02\%$

$$LLCI = [ p_1 - p_2 ] - 1.645 * \text{sqrt}[ p*(1-p)/n_1 + p*(1-p)/n_2 ]$$

$$LLCI = [0.373 - 0.220] - 1.645 * \text{sqrt}[0.289(1-0.289)/83 + 0.289(1-0.289)/100] = 0.042,$$

which is positive and suggests that the experimental product is superior over the positive control in reducing the incidence of new infections by at least 4.2%.

Sample size (n) to detect a difference between two treatment groups. Sample size per treatment group can be calculated by the following steps proposed by Casagrande et al. (1978).

1. Determine the absolute value for a one-tailed test, and search the cumulative standard normal distribution function tables for:

$Z_1$  = critical one-tailed value for Type I error rate (usually 5%): Locate the 0.95 probability and the corresponding  $Z_1$  value, which, by interpolation is 1.645.

Z2 = critical one-tailed value for Type II error rate (usually 10%): Locate the 0.90 probability and the corresponding Z<sub>i</sub> value, which by interpolation is 1.281.

2.  $p$  (average infection rate) =  $[p_1$  (control infection rate) +  $p_2$  (experimental infection rate)]/2.
3.  $A = [ Z_1 * \sqrt{2 * p * (1-p)} + Z_2 * \sqrt{p_1 * (1-p_1) + p_2 * (1-p_2)} ]^2$ .
4.  $D$  (difference anticipated between two groups) =  $p_1$  (control infection rate) -  $p_2$  (experimental infection rate).
5.  $n = A * [1 + \sqrt{1 + 4D/A}]^2 / (4 * D * D)$ ,  
which will be the number of eligible quarters needed per treatment group.

For example (see Table 1 below), if quarters in the control group ( $p_1$ ) are expected to have a 30% infection rate (0.30), and quarters in the experimental group ( $p_2$ ) are expected to have a 20% infection rate (0.20), and average infection rate ( $p$ ) is 0.25, and the difference ( $D$ ) between infection rates is 0.10, and it is desirable to have a 90% chance of detecting a statistical difference (Type II error rate) at the 5% significance level (Type I error rate), then  $A$  is determined as follows:

$$A = [ 1.645 * \sqrt{2 * 0.25 * (1-0.25)} + 1.281 * \sqrt{0.30 * (1-0.30) + 0.20 * (1-0.20)} ]^2$$

$$A = 3.192653$$

Then determine  $n = (3.192653 * [1 + \sqrt{1 + 4D/3.192653}]^2 / (4 * D * D)) = 339$

Table 1 contains sample sizes for various infection rates.

Table 1. Number of eligible quarters per treatment group needed in comparing two proportions with a one-tailed test, assuming 90% power and 5% significance level.

Control infection rate (%)	Treatment infection rate (%)			
	5	10	15	20
30	45	76	144	339
25	62	121	292	1232
20	95	236	1027	---
15	172	787	---	---
10	513	---	---	---

## **Determining Efficacy of a Postmilking Barrier Teat Dip Based on Reduction of New Naturally Occurring Intramammary Infections**

Experimental Design. Teats of half of the quarters are dipped after each milking in the experimental barrier teat dip being tested; the other half are dipped in a positive control conventional germicide. The experimental design can be either a split-udder or a split-herd design. The numbers of new intramammary infections (IMI) in quarters with teats dipped in the experimental barrier product and in quarters dipped in the positive control dip are determined. Positive control products should previously been shown to be efficacious compared with not dipping in either experimental challenge or natural exposure trials and cited in the NMC Teat Dip Bibliography.

Selection of Experimental Herds, Cows, and Quarters. Trials should be conducted in herds in which wholehearted cooperation of managers can be attained to comply with experimental procedures. Milking equipment and milking management practices should be monitored carefully and regularly as described below to minimize machine-mediated infections. This is especially necessary in commercial herds in which constant supervision by the investigator will not be practical. All quarters are eligible for new IMI except quarters with teats that are deformed due to previous injury. Quarters with teats that are injured during the trial should be excluded for the remainder of that lactation; such quarters may re-enter the trial after a dry period if the injury has healed.

Milking System and Premilking Udder Preparation. A milking system evaluation should be performed prior to trial initiation to assure that all machinery meets the requirements set forth by the NMC Procedures for Evaluating Vacuum Levels and Air Flow in Milking Systems. Premilking udder preparation methods should be decided before the start of the trial and recorded for the final report. For studies requiring no premilking disinfection, premilking udder preparation should consist of the use of single service water-moistened towels (free of sanitizer, one per cow) to wet and clean the teats prior to fore-stripping. Fore-stripping is accomplished by expressing two squirts of milk. For studies requiring that a disinfectant be used, each quarter should be fore-stripped by expressing two squirts of milk followed by dipping of all teats with the designated premilking disinfectant. A 30-s contact time should be allowed and the teats wiped dry using a single service towel before attaching the milking unit. The premilking disinfectant used should have been previously demonstrated to be effective through NMC recommended testing and listed in the NMC Teat Dip Bibliography.

Teat Dipping. Teats of half the cows in the herd are dipped in the experimental barrier product in a split-herd design, and teats of cows in the remainder of the herd serve as positive controls. Cows should be balanced by parity, stage of lactation, and bacteriological status of quarters. The two groups of cows should be milked by the same personnel in the same facility. If two different housing conditions exist, the two treatment groups should be equally exposed to the two housing environments. Each treatment group should be identified with leg bands and/or neck chains.

When a half-udder design is used, either two diagonal teats or teats on either the right or left side of each udder are dipped with the experimental product. Teat dip is applied immediately after milking machine removal.

Sampling Schedule and Procedures. Duplicate quarter milk samples are collected to determine existing infections in the herd within 7 d prior to the beginning of the trial. A third sample is obtained when results of the first two samples differ. Single quarter milk samples are collected and cultured monthly or duplicate samples are collected and cultured bimonthly during the trial. Duplicate samples from cows collected 3 d post calving and herd additions should be cultured prior to inclusion in the trial. When any quarter develops clinical mastitis, duplicate milk samples from all four quarters should be cultured before any treatment is administered. In addition, duplicate milk samples should be collected and cultured from individual cows at drying off, leaving the study, or upon leaving the herd. All milk samples should be collected immediately before a regular milking using standard procedures (Harmon et al., 1990). Briefly, three or four streams of foremilk are discarded from each quarter before sanitizing teat ends with cotton swabs and collecting samples. Sterile swabs should be soaked in 70% alcohol from which excess fluid has been expressed; no more than two teats should be sanitized per swab. The far teats should be sanitized first and the near teats last to avoid contaminating the near teats with the sampler's fingers. When collecting milk samples, the near teats should be sampled first and the far teats last to avoid contaminating the near teats with the sampler's fingers.

Criteria for Diagnosing Infections. All milk cultures are examined bacteriologically, and the microorganisms isolated are identified according to standard procedures (Harmon et al., 1990). In determining that a quarter is free of infection when it enters the trial, no pathogens may be recovered from two of the initial duplicate or consecutive samples. An IMI is diagnosed when the same bacterial species is isolated from both of the duplicate samples taken bimonthly or from clinical quarters, or two consecutive monthly samples collected during the trial. The status of a quarter exhibiting clinical mastitis should be recorded as a bacteriologically-negative clinical case when bacteriological results of duplicate samples from a clinical quarter do not match. Clinical mastitis and IMI diagnosed during the first 7 d of lactation should not be included in data analyses of teat dip efficacy.

An individual quarter is eligible for only one infection per bacterial species during a lactation (i.e., only one *Escherichia coli* infection per quarter per lactation). Quarters infected in one lactation may be included in the trial in the subsequent lactation if it is determined that the infection was eliminated during the dry period either spontaneously or as a result of therapy.

Statistical Analyses. The purpose of the trial must be decided a priori when using a positive-control. The purpose is most often to determine if the experimental product is more efficacious than the positive-control or that the efficacy of the experimental product does not vary from that of the positive control by greater than a predetermined amount.

Environmental pathogens. To claim that an experimental barrier teat dip is efficacious against environmental pathogens (i.e., *E. coli*, *Klebsiella* spp., *Streptococcus uberis*), the efficacy of the experimental product should be greater than that of the positive control germicide against these same bacteria. To determine if the efficacy of an experimental product is greater than that of a positive control, the hypothesis is formulated and tested as if teats on positive control quarters were not being dipped. Data must express the relation between quarters becoming infected in quarters treated with the experimental teat dip and in quarters with teats dipped in the positive control product.

Differences between the percentage of quarters becoming infected between treatments can be tested with the standard normal approximation (Steel and Torrie, 1980). For a given time period, this difference is determined as follows:

Let:

$x_1$  = number new IMI in control quarters,  
 $x_2$  = number new IMI in treated quarters,  
 $n_1$  = number of eligible control quarters, and  
 $n_2$  = number of eligible treated quarters.

Then calculate:

$p_1$  (fraction of new IMI in control quarters) =  $x_1/n_1$ ,  
 $p_2$  (fraction of new IMI in treated quarters) =  $x_2/n_2$ ,  
 $p$  (pooled infection proportion) =  $(x_1+x_2) / (n_1+n_2)$ ,  
 $P$  (% reduction in new IMI) =  $1 - p_2/p_1$ , and  
 $Z = [x_1/n_1 - x_2/n_2] / \text{sqrt}[p*(1-p)/n_1 + p*(1-p)/n_2]$ .

The statistical significance of the test can then be evaluated from one-tailed standard normal tables using the  $Z$  statistic (Steele and Torrie, 1980).

An example based on total new *E. coli* IMI in dipped and control quarters in an efficacy trial (Hogan et al., 1995) using a 0.55% chlorhexidine acetate experimental barrier product (in comparison with a 1% iodophor positive control) is given below using the formula described above:

$x_1$  = number new IMI in control quarters = 19  
 $x_2$  = number new IMI in treated quarters = 9  
 $n_1$  = number of eligible control quarters = 2888  
 $n_2$  = number of eligible treated quarters = 2772

Calculate:

$p_1$  (fraction of new IMI in control quarters) =  $x_1/n_1 = 19/2888 = 0.0066$   
 $p_2$  (fraction of new IMI in treated quarters) =  $x_2/n_2 = 9/2772 = 0.0032$   
 $p$  (pooled infection proportion) =  $(x_1+x_2)/(n_1+n_2) = (19 + 9)/(2888+2772) = 28/5660 = 0.0049$   
 $P$  (% reduction in new IMI) =  $1 - p_2/p_1 = 1 - 0.0032/0.0066 = 51.5\%$   
 $Z = [(19/2888)-(9/2772)] / \text{sqrt}[0.0049(1-0.0049)/2888 + 0.0049(1-0.0049)/2772]$   
 $Z = 1.786172$

Thus, the percentage reduction in new IMI is 51.5%, and according to a  $Z$  table illustrating standard normal distribution function, a  $Z$  value of 1.76 is significant at the  $P \leq 0.05$  level, demonstrating a difference in percentage of new *E. coli* IMI between control (0.66%) and treated (0.32%) quarters.

Sample size (n) to detect a difference between two treatment groups. Sample size per treatment group can be calculated by the following steps proposed by Casagrande et al. (1978).

1. Determine the absolute value for a one-tailed test, and search the cumulative standard normal distribution function tables for:

Z1 = critical one-tailed value for Type I error rate (usually 5%): Locate the 0.95 probability and the corresponding Z<sub>i</sub> value, which, by interpolation is 1.645.

Z2 = critical one-tailed value for Type II error rate (usually 10%): Locate the 0.90 probability and the corresponding Z<sub>i</sub> value, which by interpolation is 1.281.

2.  $p$  (average infection rate) =  $[p_1$  (control infection rate) +  $p_2$  (experimental infection rate)]/2.
3.  $A = [ Z_1 * \sqrt{2 * p * (1-p)} + Z_2 * \sqrt{p_1 * (1-p_1) + p_2 * (1-p_2)} ]^2$  .
4.  $D$  (difference anticipated between two groups) =  $p_1$  (control infection rate) -  $p_2$  (experimental infection rate).
5.  $n = A * [1 + \sqrt{1 + 4D/A}]^2 / (4 * D * D)$ ,  
which will be the number of eligible quarters needed per treatment group.

For example (see Table 1 below), if quarters in the control group ( $p_1$ ) are expected to have a 20% infection rate (0.20), and quarters in the experimental group ( $p_2$ ) are expected to have a 10% infection rate (0.10), and average infection rate ( $p$ ) is 0.15, and the difference ( $D$ ) between infection rates is 0.10, and it is desirable to have a 90% chance of detecting a statistical difference (Type II error rate) at the 5% significance level (Type I error rate), then  $A$  is determined as follows:

$$A = [ 1.645 * \sqrt{2 * 0.15 * (1-0.15)} + 1.281 * \sqrt{0.20 * (1-0.20) + 0.10 * (1-0.10)} ]^2$$

$$A = 2.16498$$

Then determine  $n = (A * [1 + \sqrt{1 + 4D/A}]^2 / (4 * D * D))$

$$n = (2.16498 * [1 + \sqrt{1 + 4(0.10)/2.16498}]^2 / (4 * 0.1 * 0.1)) = 237$$

Table 1 contains sample sizes for various infection rates.

Table 1. Number of eligible quarters per treatment group needed in comparing two proportions with a one-tailed test, assuming 90% power and 5% significance level.

Control infection rate (%)	Treatment infection rate (%)			
	5	10	15	20
30	45	76	144	339
25	62	121	292	1232
20	95	236	1027	---
15	172	787	---	---
10	513	---	---	---

The length of a trial to demonstrate acceptable efficacy of a teat dip depends on: 1) number of quarters tested; 2) rate of new IMI in dipped quarters; and 3) percentage reduction of IMI in dipped quarters. The greater the amount by which the mean reduction exceeds the minimum acceptable, the smaller the size of trial needed to determine efficacy. For groups of a given size, the number of quarters required to become infected in the control group varies depending on the extent to which the dip is successful in reducing the incidence of infection in the dipped group.

Contagious pathogens: The efficacy of an experimental barrier teat dip against *Staphylococcus aureus* and *Streptococcus agalactiae* should not be less than that of the positive control teat dip. Experimental products may be tested to determine if efficacy is "equal" to that of the positive control, and equivalence can be evaluated by constructing a confidence interval on the difference between two proportions.

Statistical Analyses. A 95% one-sided lower limit (LLCI) confidence interval for the difference between proportions can be computed using the normal approximation and the critical one-tailed Z of 1.645. For a given time period, this difference is determined as follows:

Let:

x1 = number new IMI in control quarters,  
 x2 = number new IMI in treated quarters ,  
 n1 = number of eligible control quarters, and  
 n2 = number of eligible treated quarters.

Then calculate:

p1 (fraction of new IMI in control quarters) =  $x1/n1$ ,  
 p2 (fraction of new IMI in treated quarters) =  $x2/n2$ ,  
 p (pooled infection proportion) =  $(x1+x2) / (n1+n2)$ ,  
 P (% reduction in new IMI) =  $1 - p2/p1$ , and  
 $Z = [ x1/n1 - x2/n2 ] / \text{sqrt}[ p*(1-p)/n1 + p*(1-p)/n2 ]$ .

LLCI =  $[ p1 - p2 ] - 1.645 * \text{sqrt}[ p*(1-p)/n1 + p*(1-p)/n2 ]$

The statistical significance of the test can then be evaluated from one-tailed standard normal tables, using the Z statistic (Steele and Torrie, 1980).

An example based on total new *Staph. aureus* IMI in dipped and control quarters in an efficacy trial (Hogan et al., 1995) using a 0.55% chlorhexidine acetate experimental barrier product (in comparison with a 1% iodophor positive control) is given below using the formula described above:

x1 = number new IMI in positive control quarters = 7  
 x2 = number new IMI in experimental product quarters = 3  
 n1 = number of eligible positive control quarters = 2888  
 n2 = number of eligible experimental product quarters = 2772

Calculate:

p1 (fraction of new IMI in control quarters) =  $x1/n1 = 7/2888 = 0.0024$

$p_2$  (fraction of new IMI in treated quarters) =  $x_2/n_2 = 3/2772 = 0.0011$   
 $p$  (pooled infection proportion) =  $(x_1+x_2)/(n_1+n_2) = (7 + 3)/(2888+2772) = 10/5660 = 0.0018$   
 $P$  (% reduction in new IMI) =  $1 - p_2/p_1 = 1 - 0.0011/0.0024 = 54.2\%$

$LLCI = [ p_1 - p_2 ] - 1.645 * \text{sqrt}[ p*(1-p)/n_1 + p*(1-p)/n_2 ]$   
 $LLCI = [0.0024 - 0.0011] - 1.645 * \text{sqrt}[0.0018(1-0.0018)/2888 + 0.0018(1-0.0018)/2772] =$   
 $-0.051$ , which is negative and suggests that the experimental product is not different from the positive control in reducing the incidence of new infections.

Presentation of Data. The report of a trial should include: 1) duration of the trial; 2) number of quarters in the trial at the onset and on the date of each monthly or bimonthly sampling; 3) number of total new IMI, categorized by bacterial species or type, that occurred in control and treated quarters; 4) the percentage differences in total new IMI between treated and control quarters and for each bacterial species; 5) the number of new clinical cases, categorized by bacteriological status, that occurred in control and treated quarters; and 6) the percentage difference in new clinical cases between treated and control quarters.

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