Examining the impact of intramammary infections with minor mastitis pathogens on the acquisition of new intramammary infections with major mastitis pathogens – a systematic review and meta-analysis

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ABSTRACT

Major mastitis pathogens such as *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus uberis, Streptococcus dysgalactiae* and the coliforms are usually considered more virulent and damaging to the udder than minor mastitis pathogens such as *Corynebacterium bovis* and coagulase-negative staphylococci (**CNS**). The current literature contains a number of studies detailing analyses with conflicting results as to whether intramammary infection (**IMI**) with the minor pathogens reduces, increases, or has no effect on the risk of a quarter acquiring a new intramammary infection (**NIMI**) with a major pathogen. In order to investigate the available scientific evidence regarding the impact of IMI with minor pathogens on the acquisition of NIMI with major pathogens, a systematic review and meta-analysis were conducted. All extant English- and French-language literature in electronic databases was searched and all publications cited by relevant papers were investigated. Results from a total of 68 studies were extracted from 38 relevant papers. Random-effects models were used to investigate the effects of CNS and *C. bovis* on acquisition of new IMI with any of the major pathogens, as well as individually for the minor pathogens and *S. aureus*. Significant heterogeneity among studies exists, some of which could be accounted for using meta-regression. Overall, observational studies showed no effect, while challenge studies showed strong and significant protective effects, specifically when major pathogens were introduced into the mammary gland via methods by-passing the teat end. Underlying risk can account for a number of unmeasured factors, and studies with higher underlying risk found more protective effects of minor pathogens. Larger doses of challenge organisms reduced the protective effect of minor pathogens, and studies with more stringent diagnostic criteria for pathogen IMI also identified less protection. Smaller studies (those utilizing fewer than 40 cows) also showed a greater protective effect than larger studies.

Keywords: major pathogens, minor pathogens, protective effect, meta-analysis

INTRODUCTION

Major mastitis pathogens such as *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus uberis, Streptococcus dysgalactiae* and the coliforms are usually considered more virulent and damaging to the udder than minor mastitis pathogens such as *Corynebacterium bovis* and coagulase-negative staphylococci (**CNS**). A number of studies conducted over the past 6 decades investigating the effects of minor pathogens on the risk of acquisition of major pathogen infections have yielded contradicting results (Green et al., 2002; Pyörälä and Taponen, 2009). Studies vary widely in methodology. Experimental challenge studies involve inoculation of quarters with both minor and major pathogens or natural infections with minor pathogens and experimentally induced infections with major pathogens. Infections have been introduced either by intra-cisternal infusion, inoculation into the teat canal or teat end exposure. Observational studies also exist that examine the interaction between natural infections with these 2 groups of pathogens in field settings.

Extreme heterogeneity exists among studies accounting for these interactions, and certain shortcomings of publications on this topic are recognized. Although a large number of mastitis pathogens exist in nature, only a few strains of pathogens are typically tested in laboratory settings. Also, one of the roles of minor pathogens may be to prevent entry of major pathogens into the mammary gland, a situation which is certainly overcome by direct infusion of pathogens into the teat canal or cistern. Definition of an IMI provides some difficulties, as even mastitis 'experts' are often in disagreement over how infection is defined (Andersen et al., 2010). Numerous issues may affect the interactions of minor and major pathogens within a quarter, cow or herd. Differences such as anatomical features, immunological characteristics, previous infections and other alterations in environment may play a role in susceptibility. Cow-level factors such as breed, season of calving, age and stage of lactation may have an effect, and herd-level factors such as overall pathogen profile undoubtedly play a role, especially when random allocation of pathogens is not used. Studies that fail to take into account these factors leave themselves open to a great number of confounding issues.

Narrative reviews bring together the diversity in results that exist in the literature (Pyörälä and Taponen, 2009; Pyörälä et al., 2011), and a number of papers have reviewed the effects of minor pathogen infections on acquisition of major pathogen NIMI, whether as a section of an all-encompassing literature review or in the introduction or discussion of a primary research article investigating such interactions. These reviews are helpful in summarizing some of what has been

shown to date, but none exist that utilize a systematic or statistical methodology in the processes of identification of relevant studies, assessment of methodology and outcomes, and synthesis of the information covered. Many of the narrative reviews in the primary research literature focus on results similar to those obtained in the trial being described, in order to support such results as having been previously identified. With such diversity in findings, however, this is almost always possible to some degree, even when the authors are citing trials conducted 50 years previous and involving only a handful of subjects. A systematic review, on the other hand, uses a transparent method to identify relevant literature, extract the required information and summarize and synthesize the results of the included studies (Sargeant et al., 2006). Qualitative methods can be used to investigate the differences or similarities between studies, while quantitative methods seek to combine study results and/or investigate factors contributing to heterogeneity among studies. Due to the diverse publications and previous research concerning the effect of minor pathogens in bovine mastitis, a systematic review and meta-analysis was selected to shed further light on the information available on this topic. The objective of this paper was specifically to investigate the impact of minor pathogen IMI on the acquisition of new major pathogen IMI in the bovine udder. Because substantial heterogeneity among studies was expected, the specific aim was to quantify and describe the sources of heterogeneity rather than provide an overall estimate of the effect of minor pathogen IMI on major pathogen NIMI.

MATERIALS AND METHODS

Literature Search

An online literature search was conducted, consulting PubMed, CAB Abstracts, Agricola and Google Scholar. To identify papers referring to minor pathogens, keywords such as *minor*, *Corynebacter** and *coagulase-negative staphylococc** were used. In order to include papers referencing major pathogens, *major*, *Staph* aureus*, *Staph*, Strep** and *coliform** keywords were also used. These were then combined (using *AND* statements) with the keywords *mastitis*, *risk* and *protect** in order to identify studies investigating these interactions. Studies in English or French were considered, and were drawn from peer-reviewed journals, conference proceedings, book chapters and theses. Upon initial reading of salient articles by the primary author, work cited in the 'References' section that appeared to deal with the interactions between minor and major pathogens were also retrieved.

Additionally, 19 investigators listed as authors on papers addressing this topic were contacted in order to identify any unpublished or difficult to locate studies related to the question of interest.

Only one additional narrative review was identified by this route. Only manuscripts relating to mastitis pathogens of the bovine were included, and papers needed to specifically deal with IMI caused by minor pathogens (as opposed to teat apex colonization, for instance) and NIMI caused by major pathogens (as opposed to clinical mastitis without delineation of pathogen type, etc.).

Data Extraction

A set of 3 structured data collection forms (based on Sargeant et al., 2006) were devised and used for extraction of information. One form addressed general information given in the paper, a second included specific questions relating to the study type described, and a third form provided a structure for extraction of the outcomes and raw data given in the manuscripts. Two independent investigators read and extracted information simultaneously. The data extraction forms were initially tested on 5 included papers and changes for clarity were addressed. Any differences in data extraction were discussed by the investigators, and consensus was reached after further referring to the paper in question. If a paper reported more than one set of observations for separate minor or major pathogens, each pairing of pathogens was reported separately. If a paper reported the effect of a minor pathogen on a combined group of major pathogens (e.g. CNS vs. *S. aureus, S. uberis and S. dysgalactiae*), or when raw data were presented that allowed this grouping to be performed, these data were also extracted independently. Although results from studies reporting by treatment groups (e.g. lactational therapy groups, post-milking teat disinfection trials, etc.) were recorded separately when details were provided in the manuscript, these were often combined in the meta-analysis due to inadequate group sizes for comparison.

Descriptive, study design and study quality information extracted from each study are listed in Table 1. Challenge studies and observational studies also had individual information relevant to their study design extracted.

If presented in individual manuscripts, quarter-level data were used to construct 2x2 tables for use in the meta-analysis. These raw data were available for all but 2 papers detailing challenge experiments and all but 8 papers reporting observational findings. Four manuscripts presented odds ratios (**OR**) - one unadjusted from a case-control analysis (Lam et al., 1997), and 3 adjusted from multilevel models (Green et al., 2002; Green et al., 2005; Schukken et al., 1999) – and either standard errors (**SE**) or 95% confidence intervals, from which SE could be calculated. Three references (Hogan et al., 1988; Woolford et al., 2001; Zadoks et al., 2001a) reported rates of new infections, and others reported differing measures such as median difference of infection at the herd level (Michel et al., 2011), clinical differences (Spencer et al., 1968), and coefficients from Poisson models (Zadoks et al.,

2001b). Data from these last 6 papers were not able to be included in the meta-analysis, although, as selections for the systematic review, reported outcomes are discussed later in this manuscript.

A number of manuscripts presented results for multiple minor and major pathogens, so individual study values were constituted using the raw data or reported OR. Studies from observational papers representing a number of different data collections (on lactating cows versus dry cows, for instance) were represented separately. Exceptions to this occurred when there appeared to be no substantial differences between the trials or the researchers themselves combined data in the original publication. Many challenge studies reported differing pathogens and amounts of pathogens used in challenges; these data were extracted as separate studies where sufficient detail was reported.

Meta-analyses

A number of meta-analyses were carried out in order to investigate the effect of minor pathogen IMI on the acquisition of NIMI with major pathogens (Table 2).

- Manuscripts differed in the individual pathogens investigated, so a general meta-analysis comparing the effect of both minor pathogens (CNS and *C. bovis*) on acquisition of NIMI with any major pathogens (*S. aureus, S. agalactiae, S. uberis, S. dysgalactiae, Escherichia coli*, and other pathogens such as *Klebsiella spp.* or environmental streptococci) was initially performed.
- Studies were then grouped as observational or challenge studies, and a meta-analysis within each group was performed.
- Separate meta-analyses investigating the effects of CNS and *C. bovis* individually on all major pathogens were also performed.
- The effects of each minor pathogen on acquisition of NIMI with *S. aureus* were also investigated in separate meta-analyses.

Due to identification of a high degree of heterogeneity among studies, random effects meta-analyses using the method described by DerSimonian and Laird (1986) were performed. Odds ratios were selected as the measure of association in order to incorporate the results of the 4 papers not containing raw data but reporting OR and SE or 95% confidence intervals. In order to calculate OR for those 2x2 tables with cells containing the value ‘0’, an empirical continuity correction was employed, as this is expected to decrease the amount of bias in estimation of effect in the subsequent meta-analyses (Sweeting, et al., 2004).

Meta-regressions

Meta-analysis regressions, or meta-regressions, were employed to investigate the reasons for heterogeneity among studies. These analyses use data summarized at the study level to fit regression models with the natural log of the OR (**lnOR**) as the outcome variable.

- Firstly, all predictors described in Table 1 were investigated for their contribution to heterogeneity using univariable meta-regressions.
- Next, predictors of interest as well as those showing association ($P \leq 0.05$ before Bonferroni correction) with the lnOR were combined in multivariable meta-regressions when the number of studies available made this possible.
- Separate meta-regressions were fit for datasets involving any minor pathogen and any major pathogen, observational studies only, challenge studies only, CNS and major pathogens, *C. bovis* and major pathogens, CNS and *S. aureus*, and *C. bovis* and *S. aureus.*

There were often such a small number of studies included – especially in the individual CNS and *C. bovis* effects on *S. aureus* meta-regressions – that multivariable meta-regressions were not possible. Factors were initially combined with the 3-level study design variable and assessed for further significance. Those that appeared to provide much the same information as study design (collinear) or were expected to be caused by study design (intervening) were not included in further modeling (Table 1). Multivariable models were then built using a manual stepwise procedure in an attempt to account for the most between-study heterogeneity while maintaining statistical significance ($P \leq 0.05$) in the predictors. Factors within the multivariable model were also assessed for collinearity to make sure they were not representing the same information. Adjusted-R^2 values were used to indicate the relative reduction in the between-study variance (Harbord and Higgins, 2008). In order to avoid Type I errors occurring due to inclusion of multiple predictors in the multivariable meta-analyses, a random permutation test based on Monte Carlo simulation was used to provide a multiplicity adjustment of the P-values (Harbord and Higgins, 2008). To provide sufficient precision, the command was set to 20,000 permutations, and results were compared to those obtained in the multivariable models.

Publication Bias or Small-Study Effects

A funnel plot was generated in order to evaluate the presence of publication bias or small-study effects. This plot sets the odds ratio against its standard error, thereby displaying the differences among effects of smaller studies and those of larger studies (Harbord et al., 2009). Lack of symmetry in the

plot suggests that bias may be present, or that small studies with large standard errors may be influencing the estimate of the effect size. Egger's test for asymmetry was chosen from among the possible options and applied to evaluate the evidence for publication bias or small-study effects (Harbord et al., 2009). All analyses were conducted using Stata 12IC (StataCorp, College Station, TX).

RESULTS

Literature Search

The literature search identified 267 abstracts which were further investigated for relevance to the topic. After removal of manuscripts that did not include information relating to infection with minor and major mastitis pathogens, did not specify bacteriological interaction, reported effects of minor pathogens without any information regarding major pathogen IMI, or did not directly address the specific question under investigation (Figure 1), 38 manuscripts containing the results of 69 studies met the selection criteria for inclusion in the meta-analysis (Appendix 1). Of these, 35 were published in peer-reviewed journals, 2 came from conference proceedings, and one was a thesis.

Descriptive Information

The papers included represented a wide range of research conducted over 6 decades in a number of countries. Descriptive information here is provided for all papers, while information related specifically to the studies included in the meta-analysis is listed in Table 1. Papers often contained a number of trials or observations about different minor and major pathogens. Manuscripts reported results of analyses involving what they termed CNS, coagulase-negative micrococci or simply micrococci. These have been combined here and are referred to as CNS. Twenty-nine papers contained studies examining minor pathogen effects on NIMI with *S. aureus* (12 with CNS only, 11 with *C. bovis* and 6 with both), while 13 papers contained studies with *S. agalactiae* as the outcome (6 with CNS, 6 with *C. bovis* and 1 with both). Fifteen papers presented results of major infections with coliform bacteria (5 with CNS, 6 with *C. bovis*, 4 with both). Studies were conducted in the UK (n=9), the US (n=8), Sweden (n=5), Canada (n=5), France (n=3), the Netherlands (n=3), New Zealand (n=2), Denmark (n=1), Finland (n=1), and Switzerland (n=1). Holstein cattle were mainly used, followed by Jersey cattle and other dairy breeds (Swedish Red and White, Guernsey, Friesian, Meuse-Rhine-Yssel). Total number of cows was often small for challenge studies (median=19, mean=64.5), although one multi-year challenge trial included 600 cows (Nickerson and Boddie, 1994), and papers often detailed

the results of a number of challenge studies conducted on subsets of cows. Observational studies were much larger in terms of total cows (median=255, mean=673.4). A variety of definitions for IMI were found among the papers, with diagnostic criteria from 20 cfu/mL to 350 cfu/mL and some papers requiring that IMI be present in consecutive samples, duplicate samples, or in samples from cows diagnosed with clinical mastitis in order to be considered infected. These were categorized into 4 variables, 2 each for minor and major pathogen definitions: a threshold category (<100 cfu/mL, 100-299 cfu/mL and ≥300 cfu/mL) and a diagnosis classification (whether or not IMI needed to be diagnosed in consecutive, duplicate, or clinical mastitis samples). A subjective assessment of whether the conclusions of the authors were supported by the results presented in the paper was made by the data extractors.

All but one manuscript describing challenge experiments were included in the meta-analysis. Spencer et al. (1968) gave a general impression that no effect of minor pathogens was elicited, but did not report any data that were usable in further analyses. Of the remaining 16 papers reporting challenge experiments, 37 studies were compiled. Of these, challenge studies were separated into categories: 'challenge-challenge' studies where quarters were challenged with a minor pathogen and then challenged with a major pathogen (n=17), 'natural-challenge' studies where quarters were naturally infected with minor pathogens before being challenged with major pathogens (n=9), and 'both-challenge' studies where some quarters were naturally infected with minor pathogens and some were challenged with the same minor pathogen (to increase numbers in the minor-pathogen IMI group), then all were challenged with a major pathogen (n=9) (Figure 1). There were 2 studies that included quarters challenged with a minor pathogen (CNS) and then observed for natural infection with a major pathogen (Pankey et al., 1985), but these were combined with the observational study group, as challenge with a major pathogen was believed to be the basis for an experimental challenge study (Figure 1). Twenty-two of the challenge studies involved CNS as the minor pathogen, and 15 challenge studies investigated the effects of *C. bovis*.

Of the 21 papers detailing observational work, 5 did not contain data that could be used in the meta-analysis (Figure 1). Hogan et al. (1988) reported rates of infection with minor and major pathogens, but extracting raw data from the information given was not possible. Michel et al. (2011) included only median differences of minor and major pathogen infections between herds and estimated a protective effect based on herd profile. Woolford et al. (2001) summarized a higher incidence of *S. uberis* infection in quarters infected with *C. bovis*, but did not provide any information with which to extract any further data. Zadoks et al. (2001b) reported a higher incidence of *S. uberis* in quarters infected with other pathogens (including minor pathogens) and also reported parameters in Poisson

models (2001a), but this information could not be transformed into a format for meta-analysis. The other 16 manuscripts comprised 31 usable individual studies. Of these, 11 reported on the effects of CNS, and 20 represented the effects of *C. bovis* IMI.

In total, 69 studies were extracted from the 38 manuscripts. One study failed to find any major pathogen NIMI, and as such was not expected to contribute to the overall meta-analysis (Sweeting et al., 2004), so it was removed, leaving 68 usable studies (Figure 1).

Meta-analyses

Due to the extreme heterogeneity among studies in these meta-analyses, the values obtained for the estimates of overall OR cannot be expected to accurately represent the effect of minor pathogen IMI on the acquisition of NIMI with major pathogens. Overall measures of effect are reported, but readers are cautioned that these overall measures may be of limited use due to the substantial differences among the studies included in these meta-analyses.

The estimated effects of minor pathogen IMI on acquisition of major pathogen NIMI are given in Table 2 and are illustrated in forest plots in Figures 2, 3 and 4 (separated by type of study). A significant protective effect was seen for the effects of all minor pathogens on acquisition of NIMI with all major pathogens. Since study design was expected to be a major contributor to the heterogeneity among studies, it was broken down into subgroups and analyzed separately. A significant protective effect was present in challenge studies; there was no effect seen amongst observational studies (Table 2). There was considerable between-study heterogeneity (τ^2), most notably in the 'challenge-challenge' studies (Table 2, Figure 3).

A sensitivity analysis was conducted on the full dataset by removing studies individually and assessing change in effect. No one study was influential enough to alter the OR estimate from the statistically significant protective range (data not shown). Removal of all 12 studies by the research group of Linde et al., however, shifted the overall OR estimate to 0.84 (95% CI=0.65, 1.10), a statistically non-significant effect (P=0.20).

Meta-regressions

A specific aim of this research was to describe and quantify the heterogeneity among studies that exists in the literature in order to identify areas of difference that might explain the divergent effects of minor pathogens reported through the years. Univariable meta-regressions revealed that a number of predictors had significant influence on the OR estimates, even when a Bonferroni correction was applied (Table 1).

Minor Pathogens and Study Design. There was little evidence of the presence of either CNS or *C. bovis* contributing to the heterogeneity in OR among studies in the dataset including both minor pathogens (P=0.09, adjusted R^2=-0.4%), therefore this variable was not included in further multivariable meta-regressions. Overall, ORs in challenge studies were significantly lower than those found in observational studies (P=0.01). In order to further delineate the nature of this relationship, the categories of challenge studies were separated; this increased the R^2 to 37.9%, and showed that the majority of the difference in effect came from the 'both-challenge' studies, those where quarters were challenged with minor pathogens or could be naturally infected with minor pathogens before major pathogen challenge. Studies in the 'both-challenge' category were significantly more likely to show a protective effect of minor pathogens (P<0.001) compared to observational studies; compared to no effect (OR=1), 'both-challenge' studies were more likely to show substantial protection (OR=0.06). Studies in the 'natural-challenge' category were also significantly more likely to show protection (P=0.04, protection of OR=0.33 compared to no effect), while the ORs of 'challenge-challenge' studies were not predicted to be significantly different from those generated by observational studies. Ten of the 11 studies in the 'both-challenge' group were conducted by one research group (Linde et al.), however, and the overall methods of this research group were believed to account for the majority of these differences. Since all of these studies had animals that were challenged with a minor pathogen, these 11 'both-challenge' studies were combined with the 'challenge-challenge' group to form a 3-level categorical variable for study design (observational, 'challenge-challenge' and 'natural-challenge'). As this set of variables was believed to confound the relationship between the outcome and a number of other predictors in the analyses, this representation of study design was forced into all multivariable meta-regression models.

Underlying Risk. Underlying risk refers to risk of the outcome (here, a NIMI with a major pathogen) in the population of bovine quarters not infected with minor pathogens, and accounts for a number of unmeasured variables in a population. There was significant variation in the underlying risk among studies - 48 different values ranging from an odds of 0.01 to 36.2 were calculated - and underlying risk was a significant predictor in unconditional analysis. Since underlying risk is inherently related to the OR, the use of meta-regression alone for evaluation of its contribution to heterogeneity is not adequate (Dohoo et al., 2007). To further evaluate the effect of heterogeneity due to underlying risk, a recommended maximum likelihood random slopes model was fit and compared to the outcomes from standard meta-regression. This model resulted in a coefficient estimate (β) very close to the value of β estimated by standard meta-regression (β=-0.45, SE=0.06 in recommended model, β=-0.50, SE=0.06 in meta-regression). Since this value was in close agreement and was not

much less than 0, the bias in estimation of the outcome and of β by standard tools was assumed to be small, and the use of standard meta-regression was deemed sufficient (Dohoo et al., 2007).

As underlying risk in a study increased, the amount of protection due to minor pathogens estimated by that study also increased. Underlying risk was seen to be much lower in observational studies (mean odds of major pathogen NIMI in quarters not infected with minor pathogens = 1.8) as opposed to 'challenge-challenge' studies (mean odds = 4.5) or 'natural-challenge' studies (mean odds = 10.0). Underlying risk was not significantly associated with the outcome in observational studies. Underlying risk was a significant predictor in challenge studies, however, with a similar level of effect in both 'challenge-challenge' and 'natural-challenge' studies.

Unconditional Analyses of Other Factors. A number of study-level factors proved to be significant predictors of the heterogeneity among studies in unconditional analyses. Because of the multiple comparisons made, Bonferroni correction should be applied in order to decrease Type I error, and P-values were divided by 34 in order to evaluate significance (only factors with P-values <0.0015 would be considered statistically significant). A number of predictors would have been significant after Bonferroni correction or were close enough to significance to be of specific interest. Table 1 provides an overview of the factors investigated, their unconditional associations with the outcome and the amount of variation explained by each factor.

Briefly, the odds of a study finding an increased risk of major pathogen NIMI caused by minor pathogen IMI increased through the years, and the season in which the study took place was significant, although the major difference was among studies performed over all 4 seasons and those that did not declare in which season they were performed (mainly those that were short trials). These shorter studies were more likely to find a protective effect of minor pathogens than were studies conducted over a longer period of time. Studies conducted on a combination of research and commercial farms found significantly more risk of minor pathogen IMI than studies conducted either on commercial farms, and there were no significant differences among studies conducted on research farms and those conducted on commercial operations (P=0.07), although there was a trend for studies on research facilities to find protection. Studies that took samples more often were more likely to find a protective effect of minor pathogens. Requiring that minor or major pathogen IMIs be found in duplicate samples, consecutive samples or in samples from cows diagnosed with clinical mastitis (a more stringent requirement for IMI diagnosis than just finding it in single samples) was associated with an increased likelihood to identify minor pathogen IMI as risk factors. Studies that failed to report loss of study subjects to follow-up were also more likely to find increased risk associated with minor pathogen IMI than were studies that did report loss to follow-up (P=0.001).

Multivariable Model. Eighteen of the 34 factors assessed by unconditional analyses had P≤0.05 before Bonferroni correction, so were investigated further in multivariable meta-regressions (Table 1). A multivariable model with study design, minor pathogen IMI definition and breed was built which explained 58.0% of the between-study heterogeneity (τ^2 reduced from 1.67 to 0.70; Table 3). 'Challenge-challenge' studies were significantly more likely to find protection than observational studies, as were 'natural-challenge' studies. Studies where minor pathogen IMI was only diagnosed if the minor pathogen appeared in duplicate samples, consecutive samples or in samples from quarters with clinical mastitis (as opposed to single samples) were significantly more likely to show increased risk of major pathogen NIMI by minor pathogen IMI. A similar effect was also seen for studies in which major pathogen NIMI was stringently defined, but these 2 variables were very collinear and the effect was greater for minor pathogens, so the minor pathogen variable was included in the multivariable model. In order to avoid Type I errors occurring due to inclusion of multiple predictors in the multivariable meta-analysis, a random permutation test based on Monte Carlo simulation was used to provide a multiplicity adjustment of the P-values (Harbord and Higgins, 2008). To provide sufficient precision, the command was set to 20,000 permutations and results were compared to those obtained in the multivariable model. 'Challenge-challenge' and 'natural-challenge' studies remained significantly different from observational studies (P=0.001 and P=0.04 respectively), and diagnosis of minor pathogen IMI requiring duplicate, consecutive or clinical mastitis was still significantly different from diagnosis on single samples alone (P=0.009). The P-value for the increased risk for studies conducted on Jerseys as compared to Holsteins became borderline significant (P=0.07).

Underlying risk was not included in the above model as it was believed to be an intervening variable, coming between the role of study design and the outcome (lnOR) on the causal pathway. In order to build a model representing the role study design played, therefore, underlying risk could not be included. Underlying risk was, however, believed to account for much of the heterogeneity in the outcome, so adding it to a multivariable model was of interest. Study design also needed to be included in such a model as it was believed to have a confounding effect on the underlying risk. Another multivariable model was built including underlying risk, therefore, that was able to account for 81.7% of heterogeneity among studies (data not shown). This model was similar to the model presented in Table 3, but breed was found to be associated with underlying risk in simple linear regression, so was not included. The true effect of study design was obscured in this model, although the effect of minor pathogen IMI diagnosis was relatively unchanged. This model revealed that, as the risk of a major pathogen NIMI in quarters not infected with a minor pathogen increased, the odds of such a study

identifying overall risk for major pathogen NIMI decreased (i.e. studies with higher underlying risk found more protective effects of minor pathogens; OR 0.61 compared to no effect, P<0.001).

Factors Specific to Observational and Challenge Studies. Because of their intrinsic differences, certain factors relating specifically to challenge studies and observational studies were evaluated separately. Studies where cows were housed in conditions similar to those in the field were more likely to identify risk caused by minor pathogens than those that did not describe housing conditions, but this result was not significant when a Bonferroni correction was applied, nor was it significant in further multivariable model building. This indicates that differences among observational studies can be mostly explained by factors that were common to both observational and challenge studies (Table 1). A number of exclusive predictors were significant for challenge studies, however, and in unconditional analyses appeared to explain a significant portion of the heterogeneity among studies. Studies that included cows naturally infected with minor pathogens in challenge studies were more likely to identify a protective effect of minor pathogens than studies using uninfected cows (P<0.001). Studies that did not report whether or not they tested cows prior to major pathogen challenge were also more likely to find a protective effect of minor pathogens (P=0.03). Route of administration of minor and major pathogens was also significantly associated with the outcome. Studies where minor or major pathogens were administered via immersion of the teat in an infective broth (teat dip) reported less protection than those involving direct infusion of minor pathogens into the teat (intra-cisternal injection or cannulation into the teat cistern or teat duct) (P<0.001). As the interval between minor pathogen diagnosis and major pathogen challenge increased, the amount of protection afforded by minor pathogen IMI decreased (P<0.001).

Predictors that were significant (P≤0.05) in unconditional analyses (before Bonferroni correction) were evaluated for collinearity with study design using tabulation for categorical predictors and simple linear regression for continuous predictors. All predictors that were not collinear with study design retained their significance (Table 1). These predictors were evaluated for collinearity with one another, then entered into multivariable models in a stepwise fashion in order to achieve a maximum of R^2 while maintaining statistical significance for the predictors. A model explaining 66.3% of the heterogeneity between studies resulted, which contained study design, major pathogen dose, and whether or not a study adequately reported the challenge protocols (Table 4). Study design was not significant in unconditional analyses among challenge studies, nor was it significant in the multivariable model. In studies utilizing major pathogen >500 cfu/mL doses, the amount of protection afforded by minor pathogen IMI was decreased (lnOR increased). Studies that did not report the dose of major pathogen used in experimental challenge also showed a decreased amount of protection

compared to studies that used the smaller dose. Studies that did not adequately report challenge protocols were all smaller studies (<40 cows) and showed overall more protective effect of minor pathogens on acquisition of NIMI with major pathogens (lnOR decreased). The random permutation multiplicity adjustment was also applied to this model at a setting of 20,000 permutations, and the P-value for the reporting of challenge protocols remained significant (P=0.02), as did the difference seen between non-reporting of major pathogen challenge dose vs. the lower dose (P=0.005).

Data Subsets. Many of the same factors were significant in meta-regressions for the data subsets (CNS and all major pathogens, *C. bovis* and all major pathogens, CNS and *S. aureus, C. bovis* and *S. aureus*), although it was more difficult to build multivariable meta-regression models for these subsets due to the smaller numbers of studies. Of note was that the threshold for diagnosis with either a minor pathogen or major pathogen was a significant predictor of the outcome in the data subset for CNS with all major pathogens, even when modeled with study design. These 2 variables accounted for 44.0% of the heterogeneity between studies. Similar to previous results, challenge studies showed more protection than observational studies. Studies that used a liberal diagnosis for IMI or NIMI (<100 cfu/mL) were more likely to identify a protective effect of minor pathogens (OR decreased to 0.30 compared to no effect, P=0.08) than those using ≥100 cfu/mL, as were studies that did not specify the threshold used for diagnosis with IMI (OR decreased to 0.18 compared to no effect, P=0.01).

Publication Bias or Small-study Effects.

Funnel plots were generated and used to visually assess the evidence for publication bias or small-study effects (Figure 5). Study size (and therefore study standard error) was confounded by study type, so observational studies and challenge studies were assessed separately. The graph for observational studies looked roughly symmetrical (data not shown), and the Egger's test for funnel plot asymmetry gave little evidence for small study effects (P=0.31). Challenge studies were distributed more on the left side of the graph (Figure 5), however, with 2 small studies showing protective effects of minor pathogens and large standard errors being evident in the left lower quadrant. No small studies showing increased risk of minor pathogens were seen in the corresponding right lower quadrant. Studies of moderate size were identified in the right middle of the plot, but substantially more moderately-sized studies existed in the left middle. The Egger's test indicated evidence for small study effects (p=0.04) when only challenge studies were assessed. An attempt to impute estimates for types of studies that might be missing from the literature review ('trim and fill' method) resulted in no changes to the data (Steichen, 2000).

DISCUSSION

A wide variety of literature pertaining to minor and major mastitis pathogen interaction exists. The studies selected for this meta-analysis, however, pertain specifically to minor pathogen IMI and its effects on the acquisition of major pathogen NIMI. During the literature search, a number of papers addressing questions of somatic cell count differences, duration of major pathogen infections in quarters previously or concomitantly infected with minor pathogens, morphological changes in the mammary gland after minor and major pathogen infections and the effects of minor pathogens not specifically causing IMI (e.g. on teat apices) were also identified. Although research in these areas also provides insight into the effects of minor pathogens in the bovine udder, these studies did not address the specific research question set for the analyses presented here. As such, these types of investigations and the information they contain can be used to augment work such as that presented here to provide further insight into the interactions between minor and major pathogens.

Meta-analyses

It is emphasized again that, due to the large amount of heterogeneity among these studies, estimation of an overall effect of minor pathogens has limited utility. The meta-analyses presented here do suggest overall, however, that minor pathogens have a protective effect against NIMI with major pathogens. When broken down further, this effect was drawn from the results of challenge studies, as the result for observational studies is very close to the value for no effect and is not statistically significant (Table 2). Challenge studies, then, seem to overwhelmingly show more protection by minor pathogens, for reasons to be discussed below. Studies that could not be included in the meta-analysis because they did not report OR report differing effects, with Spencer et al. (1968), Hogan et al. (1988), Woolford et al. (2001), and Zadoks et al. (2001a, 2001b) all showing either an increase or no difference in the major pathogen infection in quarters infected with minor pathogens. The paper by Michel et al. (2011) describes higher prevalence of CNS in herds with lower prevalence of *S. aureus*, which might be inferred as protection by CNS against *S. aureus*.

The effects of the minor pathogens when separated are somewhat less clear. CNS shows a strong protective effect against the major pathogens, while for *C. bovis* this is less pronounced and does not achieve statistical significance. As many authors have suggested, there are undoubtedly differences in the effects of the minor pathogens on the differing major pathogens, although this becomes more difficult to sort out because total numbers of studies for major pathogens other than *S.*

aureus are low and, because of this low power, analyses are unable to show differences beyond what might be attributed to chance.

A number of protective mechanisms of minor pathogens have been investigated and/or suggested. Non-specific activation of the immune system through host defense mechanisms such as increase in somatic cell count and differential cell count have been investigated and play a role in the interplay between pathogens in the bovine udder (Schukken et al., 1999; Pyörälä and Taponen, 2009). Production of bacteriocins and other inhibitory substances by minor pathogens have been suggested, as have stimulation of anti-staphylococcal antibody production by the host, alteration of fatty acid concentration, and general inhibition of major pathogen passage through the teat canal (Brooks and Barnum, 1984; Nickerson and Boddie, 1994; Schukken et al., 1999; Pyörälä and Taponen, 2009). These are all plausible explanations for the protection evidenced by the results presented here, however it is beyond the scope of these analyses to suggest the reasons behind the protective effects or to lend credence to any particular theory.

Underlying Risk

Dohoo et al. (2007) have shown that, even with an underlying risk coefficient equal to -2, the amount of bias in the outcome achieved by standard meta-analysis is limited. Since the recommended maximum likelihood random slopes model gave coefficient estimates comparable to those given by standard meta-regression, this value was assumed to be accurate. Underlying risk accounted for a large amount of the heterogeneity among studies; its inclusion in a multivariable model resulted in an increase in the amount of heterogeneity explained by the model (e.g. adjusted-R^2 rose from 58.0% to 81.7%). Underlying risk may be a surrogate for the unmeasured factors contributing to variations in study populations (Dohoo et al., 2007), so its accounting for a significant proportion of the heterogeneity is not surprising. Although this paper sought to evaluate sources of heterogeneity among the included studies, there are still many factors that remain unaccounted for.

Meta-regressions

In unconditional associations (Table 1), the amount of heterogeneity accounted for by each of these predictors is substantial, with a total in excess of 100%, as many of them are representing the same information (they are collinear). Because of this collinearity, many of the predictors that were significantly associated with the outcome in unconditional analyses could not be included in the multivariable model.

Although study design was not found to account for much of the between-study heterogeneity (adjusted-R^2= 7.8%) in unconditional analysis, it was significant in the multivariable model, where both 'challenge-challenge' and 'natural-challenge' studies found more protection by minor pathogens than observational studies. It has been noted that challenge studies often bypass the natural defenses of the teat (teat orifice, keratin plug, etc.) as pathogens are infused directly into either the teat canal or teat cistern. In these studies, the majority of 'natural-challenge' studies (8/9) had major pathogens administered directly into the teat, while among 'challenge-challenge' studies there was more variation (7 into the teat, 10 by immersion and 9 that did not specify a route). The only challenge studies that identified minor pathogens as risk factors for major pathogen NIMI, however, were those that administered major pathogen via teat immersion. This was also found in unconditional analyses: studies infusing major pathogens into the teat (either by intra-cisternal injection or cannulation) identified minor pathogens as having much more of a protective effect than studies using teat immersion (OR decreased to 0.09 as compared to no effect, P<0.001) or observational studies (OR decreased to 0.15 as compared to no effect, P<0.001). This relationship also held true in the data subsets for CNS and all major pathogens (OR decreased to 0.05 compared to teat dip or observational studies, P<0.001 for both) and for CNS and *S. aureus* (OR decreased to 0.05 compared to teat dip, P=0.007; OR decreased to 0.03 compared to observational studies, P=0.001), but was borderline significant in the data subset for *C. bovis* and all major pathogens (OR for infused teats decreased to 0.18, P=0.05; observational studies showed no difference, P=0.23) and completely non-significant in the data subset with *C. bovis* and *S. aureus*. This predictor alone represented >87% of the heterogeneity among studies in both the CNS and *C. bovis* major pathogen data subsets.

It has been proposed that activity at the teat end makes the difference in penetration and eventual NIMI occurrence with major pathogens. A number of authors have suggested that minor pathogens break down teat defenses or interfere with the keratin plug, allowing major pathogens to penetrate and initiate infection (Hogan et al., 1988; Williamson et al., 1995; Zadoks et al., 2001b; Berry and Hillerton, 2002a and 2002b). It has also been shown that the presence of subclinical mastitis aids in the growth of major pathogens in the udder by the release of nutrients necessary for their survival (Mattila et al., 1984; Mattila and Sandholm, 1986; Kitt and Leigh, 1997). It may be that minor pathogens are protective against major pathogens once inside the udder, although they also may increase susceptibility to major pathogens accessing the udder tissue. If minor pathogens are indeed operating in different ways in different parts of the udder, this might explain why many challenge studies using infusion of major pathogens identify protection while challenge studies using teat immersion and observational studies (in which major pathogens must access the udder via the teat end)

often identify increased risk or no effect of minor pathogen IMI on acquisition of new major pathogen NIMI.

The protective effect of minor pathogen IMI on acquisition of major pathogen NIMI was more likely to be seen in studies that utilized a more lenient definition of when an IMI exists or when a NIMI occurs. This was evident not only in IMI definitions in the larger dataset but also in IMI threshold restrictions in the data subset with CNS and major pathogens. Stringent requirements for defining when an IMI exists or when a NIMI occurs mean that the specificity of diagnosis will be high, but at the cost of reduced sensitivity. Hence, although false positive diagnoses are minimized, false negative diagnoses will increase. Low sensitivity of bacteriological culture for the majority of pathogens has already been reported (Dohoo et al., 2011), and it is therefore possible that many of these studies underestimated the presence of minor pathogens and missed the occurrence of major pathogen NIMIs. This misclassification bias undoubtedly affected the associations presented in these studies. Since the same sampling and bacteriological methods were applied to samples from cows with and without minor pathogen infection, it is assumed that the misclassification would be non-differential and, therefore, that the associations would be biased towards the null, meaning they may be, in fact, underestimated.

Challenge studies utilizing higher doses of major pathogens for challenge and those that did not report the dosages used showed minor pathogen IMI to afford less protection against major pathogen NIMI. It is intuitive that a larger inoculum of major pathogen would more easily overcome any protective effect offered by minor pathogens, although this logic perhaps cannot be extended to studies that did not report the major pathogen dosage. Interestingly, studies without adequate descriptions of challenge protocols found more evidence for protection by minor pathogens than those with full descriptions of the protocols used. Many of these studies did not report significant details such as the route of challenge administration, method of allocation of quarters to be challenged or the time between challenge and sampling for diagnosis of NIMI. Although inadequate reporting of details such as these does not necessarily mean that studies were not conducted appropriately, it does call into question the repeatability of the studies and the overall methods by which conclusions were reached.

The majority of studies either did not report the season in which they were conducted (27/68) or were conducted over all 4 seasons (39/68). Studies not reporting the season are likely to have been conducted over a short period of time (days or weeks instead of an entire year), and were more likely to find a protective effect of minor pathogens. Studies not reporting season were also associated with more frequent sampling of quarters. Although minor pathogens have been shown to cause chronic infections in the udder (Honkanen-Buzalski et al., 1984; Pyörälä and Taponen, 2009), it may be that

they exhibit more of a protective effect over a short period of time than over long periods. Another possible explanation is that studies conducted over long periods of time may culture minor pathogens at the beginning of the study period (before dry-off, for instance) instead of identifying these pathogens very close to the time of major pathogen NIMI.

Sampling frequency was closely related to study design but, like publication year, seemed to account for a greater proportion of the heterogeneity among studies (29.3% vs. 7.8%). It is difficult to attribute an observed effect of minor pathogen IMI on major pathogen NIMI to the presence of minor pathogen IMI cultured 10-20 weeks prior to the occurrence of a NIMI, although this is what some manuscripts offered. Studies conducted over the dry period also identified minor pathogens prior to drying off and related their presence to the occurrence of major pathogen NIMI after calving, usually 6 or more weeks later. Minor pathogen infections may become chronic, but this cannot be assumed of all minor pathogen IMI during all stages of lactation, and conclusions from studies sampling over very long periods should be drawn with care.

Publication Bias or Small-study Effects

The lack of studies showing high levels of risk caused by minor pathogen IMI may be an indication of publication bias (small studies showing increased risk of minor pathogens have not been published) or of small-study effects (smaller studies showed very protective effects of minor pathogens). In Figure 5, there are only 2 small studies showing very protective effects of minor pathogens, and one small observational study also showed very protective effects (data not shown). Studies with moderate sample size and estimates of increased risk are included (right central area of the plot), although there are more moderately sized studies that show protection (left central area). There are approximately the same number of studies showing increased risk (right) in the upper portion of the plot compared to studies showing protection (left). If bias is present, however, and small or moderate studies showing increased risk of major pathogen NIMI caused by minor pathogens do exist, inclusion of these studies would serve to move the estimate of minor pathogen effect closer to the null. Another explanation is that the moderately-sized studies showing protective effects are driving the estimate of effect towards protection. The studies represented in this middle left portion of the funnel plot ranged between 4 and 40 cows. Although this relationship was not evident in the univariable meta-regression with number of cows, this may be partially because the representation of number of cows was difficult, and most closely approximated a quadratic relationship with the outcome. It can be seen in Figure 5, however, that the majority of the smaller and moderately-sized studies showed a protective effect of minor pathogens. When studies were grouped according to

number of cows (≤40 and >40), those involving more cows were seen to show more risk for minor pathogens (OR increased to 6.0 compared to no effect, P<0.001) and 35.3% of the heterogeneity among studies was explained. This parameter could not be included in multivariable models, however, since it was collinear with study design.

Although it may not be possible to conduct experimental studies on large numbers of animals, smaller studies such as these may suffer from a lack of rigorous scientific methods. In the data presented here, all challenge studies judged to have inadequate protocols were studies with fewer than 40 cows. The one study that was judged to have inadequate time between pathogen challenge and subsequent diagnosis and 2 studies that failed to describe this amount of time were also smaller studies. Smaller studies also may not utilize fully the statistical tools available to account for occurrences such as interdependence, cow-level effects or confounders. In these data, the 8 studies reporting estimates adjusted for risk factors other than minor pathogen infection were all >40 cows, as were all 6 studies where multilevel modeling was used to account for interdependence between quarters, cows and herds.

Egger's test for small study effects was significant for challenge studies, but not for observational studies. This is not surprising, as small study effects were suspected on visual interpretation of the graph of challenge studies, and it is known that all tests proposed to evaluate small study effects may give falsely positive results in the presence of extreme study heterogeneity (Harbord et al., 2009). The authors emphasize again that this excessive heterogeneity also calls into question the accuracy of the estimate of effect given by the meta-analysis.

Other Considerations

In order to maximize power and detect significant differences in the meta-analyses and meta-regressions, the 2 groups of minor pathogens and a number of major pathogen groups were combined. Although this approach makes it possible to delineate differences that may not be seen with fewer data, it certainly has drawbacks as it is not expected that these pathogens all behave in the same manner in nature. Certainly speciation of the group of pathogens referred to as CNS would be beneficial as different species exhibit a range of chronicity and inflammatory effects in the mammary gland (Supré et al., 2011). Some attempts to further examine the effects of individual minor pathogens on major pathogens were made, but resulted in non-significant outcomes, most likely due to low power.

Conclusions

In conclusion, a wide body of evidence exists on the subject of minor pathogen IMI and its effect on the acquisition of major pathogen NIMI. Significant heterogeneity among studies exists, some of which could be accounted for using meta-regression. The analyses presented here reveal that, overall, challenge studies showed strong and significant protective effects, specifically when major pathogens were introduced into the mammary gland by methods which by-passed the teat end. Observational studies were not associated with either a protective effect or increased risk of major pathogen NIMI. Underlying risk can account for a number of unmeasured factors in studies, and was significant in all challenge studies, revealing an inverse relationship with the outcome. Larger doses of challenge organisms reduced the protective effect of minor pathogens, and studies with more stringent diagnostic criteria for pathogen IMI also identified less protection. As the interval between infection with a minor pathogen and challenge with a major pathogen increased, less protection was identified, and studies in which samples were taken less frequently also demonstrated less protection. Smaller studies also showed a greater protective effect than larger studies. The data suggest that, for a number of reasons, minor pathogens might seem more protective under artificial conditions than in real-world circumstances.

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Table 1. Descriptive, study design and study quality information extracted from each of 68 studies included in the meta-analysis investigating the effect of CNS and *Corynebacterium bovis* intramammary infections on acquisition of new intramammary infections with major pathogens. Both unconditional associations from univariable meta-regressions with major pathogen infection and the association observed after controlling for study design in multivariable meta-regressions are reported.

			Unconditional[1]		**w/ study design**[2]	
Variable	**n**[3]	**Description**	**P-value**	**R^2**[4]	**P-value**	**R^2**
Descriptive information						
Publication year	68	(range: 1965-2012)	0.001[5]	21.6	Collin.[6]	
Publication source	68	Peer-reviewed, proceedings, other	0.08	5.3		
Country	68	North America, Europe, other	0.05	1.4	0.02	15.9
Breed	68	Holstein, Jersey, other	0.02	6.1	<0.0001	43.5
Season	68	Spring, winter, all, ND[7]	0.0001	28.6	Collin.	
Underlying risk (odds of disease)	60	(range: 0.01-36.2)	<0.001	69.3	Interv.[8]	
Total farms	61	(range: 1-91)	0.27	-1.5[9]		
Total cows	55	(range: 2-6825) – represented as quadratic	0.008	15.8	0.29	44.7
No. cows +minor	17	(range: 2-18)	NS[10]	--		
No. cows +major	23	(range: 2-220)	NS	--		
No. qtrs +minor	64	(range: 2-13,504)	0.40	-2.3		
No. qtrs +major	68	(range: 1-346)	0.42	-2.6		
Study design information						
Study design	68	Observational, challenge (challenge-challenge[11], natural-challenge[12])	0.04	7.8		
Minor pathogen	68	CNS, *C. bovis*	0.09	-0.4	0.26	7.6
Farm type	68	Commercial farm(s), research facility, both	0.004	20.0	Col	
Parity	68	Heifers, multiparous cows, both, ND	0.99	-8.0		
Stage of lactation	68	Dry period only, lactation only, both	0.01	13.3	Col	
Sampling frequency	60	Samples/week (range: 0.06-7.5)	<0.001	29.3	Col	
Sampling method	68	Single, duplicate samples	0.62	-3.4		
IMI definition – minor	68	Required duplicate, consecutive, or clinical mastitis for diagnosis: yes, no, ND	<0.0001	40.5	<0.0001	50.1
IMI threshold - minor	68	3 categories of cfu/mL, ND	0.04	0.2	0.02	12.6
IMI definition – major	68	Required duplicate, consecutive, or clinical mastitis for diagnosis: yes, no, ND	0.0001	27.1	<0.0001	48.9

IMI threshold - major	68	3 categories of cfu/mL, ND	0.06	0.1	0.01	14.7
Used dry cow therapy?	68	All, some none, ND, NA[13]	0.004	22.6	Collin.	
Dry cow therapy	68	Antibiotics, teat sealant, ND, NA	0.005	21.3	Collin.	
Post-milking teat dip	68	All, some none, ND	0.05	3.8	0.0006	26.9
Study quality						
Specific objective of study?	68	Yes, no	0.10	4.2		
Justified sample size?	68	Yes, no	0.03	7.8	0.09	11.3
Loss to follow-up reported?	68	Yes, partial, no	0.004	12.6	0.007	17.7
% lost to follow-up	10	(range 0-21%)	NS	--		
Conclusions supported by results?	68	Yes, no	0.36	0.4		
Effect measure	68	Unadjusted, adjusted for other predictors	0.66	-1.9		
Statistical control for clustering	68	Yes, no	0.53	-1.1		
Confounders controlled	68	Parity, stage of lactation, other, none	0.89	-5.9		
Challenge studies only						
Protocols adequately described?	37	Yes, no	0.002[14]	29.2	0.001	29.3
Experimental unit - minor	37	Some qtrs, NA	0.89	-3.5		
Experimental unit – major	37	All qtrs, some qtrs	0.92	-3.3		
Challenge allocation – minor	37	Simple random, blocked random, systematic, ND, NA	0.07	24.8		
Challenge allocation – major	37	Simple random, systematic, ND, NA	0.85	-9.3		
Inclusion of previously infected qtrs	37	All neg., neg. major, pos. minor or major, not tested	0.002	41.5	Collin.	
Challenge administration – minor	37	Intra-cisternal, cannulation into teat duct/cistern, dip, ND, NA	<0.0001	67.5	Collin.	
Challenge administration – major	33	Intra-cisternal, cannulation into teat duct/cistern, dip, ND, NA	0.0001	61.4	0.0001	57.1
Pathogen dose – minor	28	≤50,000 cfu, >50,000 cfu, ND	0.0001	60.4	Collin.	
Pathogen dose – major	37	≤500 cfu, >500 cfu, ND	0.001	47.9	0.002	39.4
Minor dx to major chal. interval	33	(range: 2-303 days)	<0.001	60.7	<0.001	60.5
Sufficient interval for dx?	37	Yes, no, ND	0.84	-4.2		
Observational studies only						
Representative housing?	31	Yes, ND	0.04[15]	13.9		

Farm selection	31	Convenience, purposive, random, ND	0.40	5.4
Indicate farm reason to decline?	31	Indicated, not indicated, single farm	0.28	4.9
Inclusion/exclusion criteria	31	Described, not described	0.30	5.6

[1]Values provided for univariable meta-regression analyses with single predictor

[2]Values provided for multivariable meta-regression analyses with predictor and 3-level variable for study design (only variables with unconditional P-values ≤0.05 before Bonferroni correction were evaluated further)

[3]Number of studies included

[4]Adjusted-R^2=value of heterogeneity between studies accounted for by this predictor (amount of variance (τ^2) accounted for by the model)

[5]34 predictors evaluated, so P-values of unconditional associations <0.0015 would be considered significant using Bonferroni method for multiple comparisons

[6]Variable represented much the same information as study design (collinear)

[7]ND = not described

[8]Variable causes the outcome but occurs between study design and the outcome (intervening)

[9]R^2 values <0 may occur when the predictor explains less variation than would be expected by chance (Harbord and Higgins, 2008)

[10]Reported by too few studies to give sensible values in meta-regression

[11]Challenge studies where quarters were experimentally challenged both with a minor pathogen and subsequently with a major pathogen

[12]Challenge studies where quarters were naturally infected with a minor pathogen and challenged with a major pathogen

[13]NA = not applicable

[14]16 predictors evaluated, so P-values of unconditional associations <0.003 would be considered significant using Bonferroni method for multiple comparisons

[15]4 predictors evaluated, so P-values of unconditional associations <0.01 would be considered significant using Bonferroni method for multiple comparisons

Table 2. Estimated effects from separate meta-analyses of minor pathogen intramammary infection (IMI) on acquisition of new intramammary infections (NIMI) with major pathogens

	n[1]	OR	95% CI	P	$\tau^{2\,2}$
Any minor pathogen/ Any major pathogen	68	0.68	0.52, 0.88	0.003	0.65
Observational studies	31	1.02	0.75, 1.39	0.89	0.46
Challenge studies	37	0.36	0.23, 0.59	<0.001	1.22
Challenge-challenge studies[3]	28	0.31	0.15, 0.66	0.002	3.11
Natural-challenge studies[4]	9	0.38	0.22, 0.67	0.001	0.25
CNS/Major pathogen	33	0.52	0.35, 0.77	0.001	0.65
C. bovis/Major pathogen	35	0.81	0.52, 1.17	0.26	0.80
CNS/*S. aureus*	21	0.57	0.33, 0.99	0.05	0.98
C. bovis/*S. aureus*	16	0.57	0.34, 0.94	0.03	0.69

[1]Number of studies included

[2]Between-study variance in OR (heterogeneity among studies)

[3]Challenge studies where quarters were experimentally challenged both with a minor pathogen and subsequently with a major pathogen

[4]Challenge studies where quarters were naturally infected with a minor pathogen and challenged with a major pathogen

Table 3. Multivariable meta-regression model to explain heterogeneity among 68 studies on the effect of minor pathogen intramammary infection on acquisition of new intramammary infections with major pathogens. The table includes odds ratios (OR), 95% confidence interval, P-values and the between-study variance, or heterogeneity (τ^2).

Variable	OR	95% CI	P	τ^2
Null model	0.55	0.38, 0.81	0.003	1.67
Multivariable model				0.70
Intercept	0.53	0.24, 1.15	0.11	
Study type				
Observational study	Baseline			
'Challenge-challenge' study	0.15	0.07, 0.35	<0.001	
'Natural-challenge' study	0.19	0.07, 0.54	0.002	
IMI definition, minor path.				
Based on single sample	Baseline			
Based on duplicate, consecutive or clinical mastitis samples	4.66	1.95, 8.50	<0.001	
Not described	1.22	0.45, 3.32	0.69	
Breed				
Holstein	Baseline			
Jersey	4.66	1.68, 12.94	0.004	
Other	0.88	0.41, 1.88	0.90	

Table 4. Multivariable meta-regression model to explain heterogeneity among 37 challenge studies on the effect of minor pathogen intramammary infection on acquisition of new intramammary infections with major pathogens. The table includes odds ratios (OR), 95% confidence interval, P-values and the between-study variance, or heterogeneity (τ^2).

Variable	OR	95% CI	P	τ^2
Null model	0.32	0.17, 0.59	0.001	2.34
Multivariable model				0.80
Intercept	0.20	0.08, 0.54	0.002	
Study type				
'Challenge-challenge' study	Baseline			
'Natural-challenge' study	1.31	0.41, 4.18	0.64	
Dose of major pathogen administered				
≤500 cfu/mL	Baseline			
>500 cfu/mL	3.71	1.27, 10.91	0.02	
Not described	11.94	3.25, 44.26	0.001	
Description of challenge protocols				
Adequately described	Baseline			
Inadequately described	0.15	0.05, 0.44	0.001	

Figure 1. Flow chart detailing inclusion, exclusion and categorization of manuscripts and studies included in the systematic review. Reasons for inclusion/exclusion are provided at each step of the systematic review.

Figure 2. Forest plot displaying a random-effects meta-analysis of the effect of CNS and *Corynebacterium bovis* intramammary infections on acquisition of new intramammary infections with major pathogens for observational studies. Lengths of horizontal lines represent 95% confidence intervals (CI) for the effect, black dots represent the individual OR estimates of the studies and gray squares are proportional to the weight given to each study. The dashed line represents the overall effect of all the studies (OR=1.02), while the solid line represents the value for no effect (OR=1). The diamond at the bottom of the dashed line represents the 95% CI for the overall effect of the observational studies (0.75, 1.39).

Figure 3. Forest plot displaying a random-effects meta-analysis of the effect of CNS and *Corynebacterium bovis* intramammary infections on acquisition of new intramammary infections with major pathogens for 'challenge-challenge' studies. Lengths of horizontal lines represent 95% confidence intervals (CI) for the effect, black dots represent the individual OR estimates of the studies and gray squares are proportional to the weight given to each study. The dashed line represents the overall effect of all the 'challenge-challenge studies (OR=0.31), while the solid line represents the value for no effect (OR=1). The diamond at the bottom of the dashed line represents the 95% CI for the overall effect of the 'challenge-challenge' studies (0.14, 0.66).

Figure 4. Forest plot displaying a random-effects meta-analysis of the effect of CNS and *Corynebacterium bovis* intramammary infections on acquisition of new intramammary infections with major pathogens for 'natural-challenge' studies. Lengths of horizontal lines represent 95% confidence intervals (CI) for the effect, black dots represent the individual OR estimates of the studies and gray squares are proportional to the weight given to each study. The dashed line represents the overall effect of all the 'natural-challenge' studies (OR=0.38), while the solid line represents the value for no effect (OR=1). The diamond at the bottom of the dashed line represents the 95% CI for the overall effect of the 'natural-challenge' studies (0.22, 0.67).

Figure 5. Funnel plot of the individual study OR estimates for the effect of CNS and *Corynebacterium bovis* intramammary infections on acquisition of new intramammary infections with major pathogens for challenge studies. Small-study effects are evidenced by the presence of numerous studies with small OR (x-axis) and large standard errors (y-axis) on the left hand side of the plot.

Figure 1.

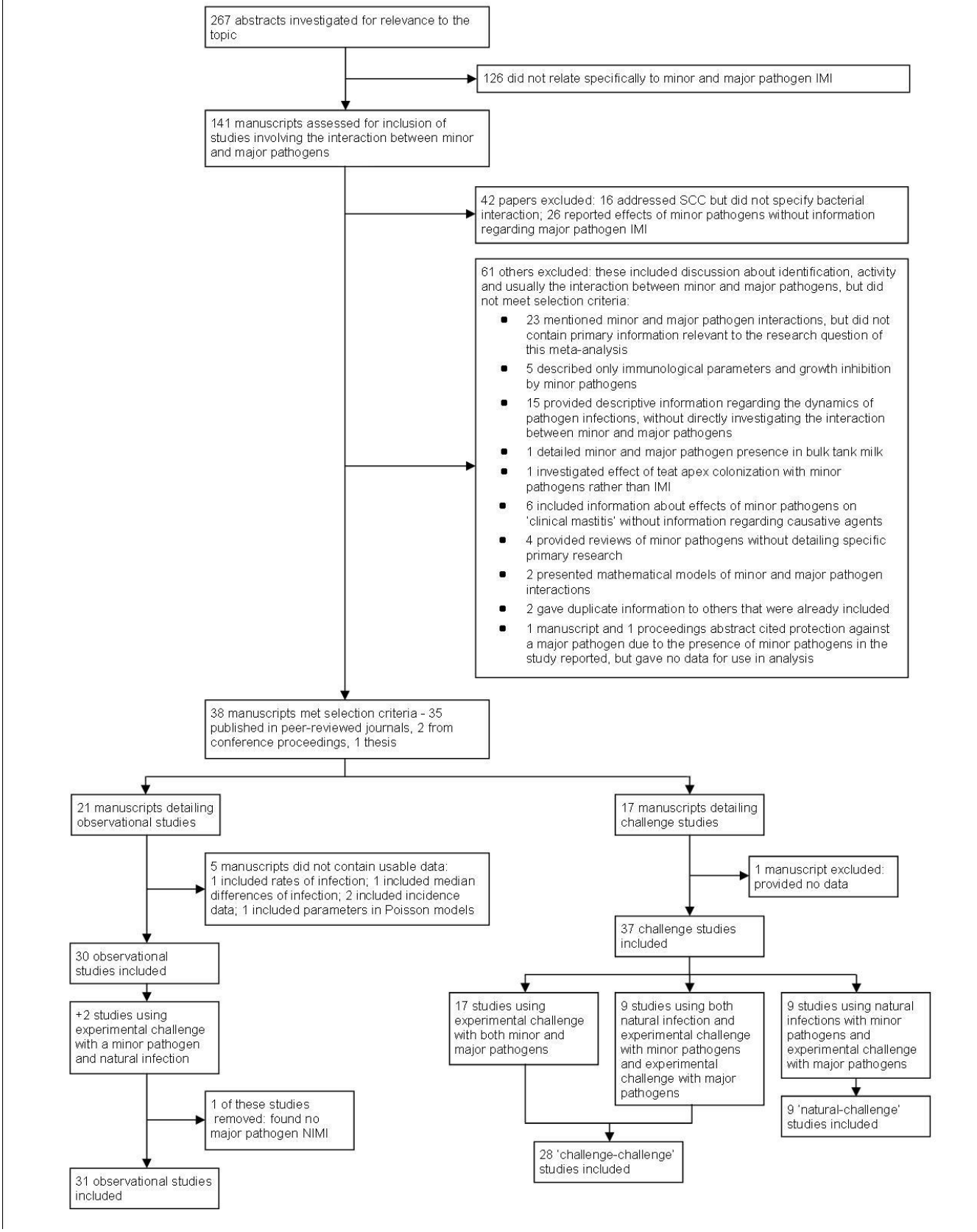

267 abstracts investigated for relevance to the topic
126 did not relate specifically to minor and major pathogen IMI
141 manuscripts assessed for inclusion of studies involving the interaction between minor and major pathogens
42 papers excluded: 16 addressed SCC but did not specify bacterial interaction; 26 reported effects of minor pathogens without information regarding major pathogen IMI
61 others excluded: these included discussion about identification, activity and usually the interaction between minor and major pathogens, but did not meet selection criteria:
23 mentioned minor and major pathogen interactions, but did not contain primary information relevant to the research question of this meta-analysis
5 described only immunological parameters and growth inhibition by minor pathogens
15 provided descriptive information regarding the dynamics of pathogen infections, without directly investigating the interaction between minor and major pathogens
1 detailed minor and major pathogen presence in bulk tank milk
1 investigated effect of teat apex colonization with minor pathogens rather than IMI
6 included information about effects of minor pathogens on 'clinical mastitis' without information regarding causative agents
4 provided reviews of minor pathogens without detailing specific primary research
2 presented mathematical models of minor and major pathogen interactions
2 gave duplicate information to others that were already included
1 manuscript and 1 proceedings abstract cited protection against a major pathogen due to the presence of minor pathogens in the study reported, but gave no data for use in analysis
38 manuscripts met selection criteria - 35 published in peer-reviewed journals, 2 from conference proceedings, 1 thesis
21 manuscripts detailing observational studies
17 manuscripts detailing challenge studies
5 manuscripts did not contain usable data: 1 included rates of infection; 1 included median differences of infection; 2 included incidence data; 1 included parameters in Poisson models
1 manuscript excluded: provided no data
30 observational studies included
37 challenge studies included
+2 studies using experimental challenge with a minor pathogen and natural infection
17 studies using experimental challenge with both minor and major pathogens
9 studies using both natural infection and experimental challenge with minor pathogens and experimental challenge with major pathogens
9 studies using natural infections with minor pathogens and experimental challenge with major pathogens
1 of these studies removed: found no major pathogen NIMI
9 'natural-challenge' studies included
31 observational studies included
28 'challenge-challenge' studies included

Figure 2.

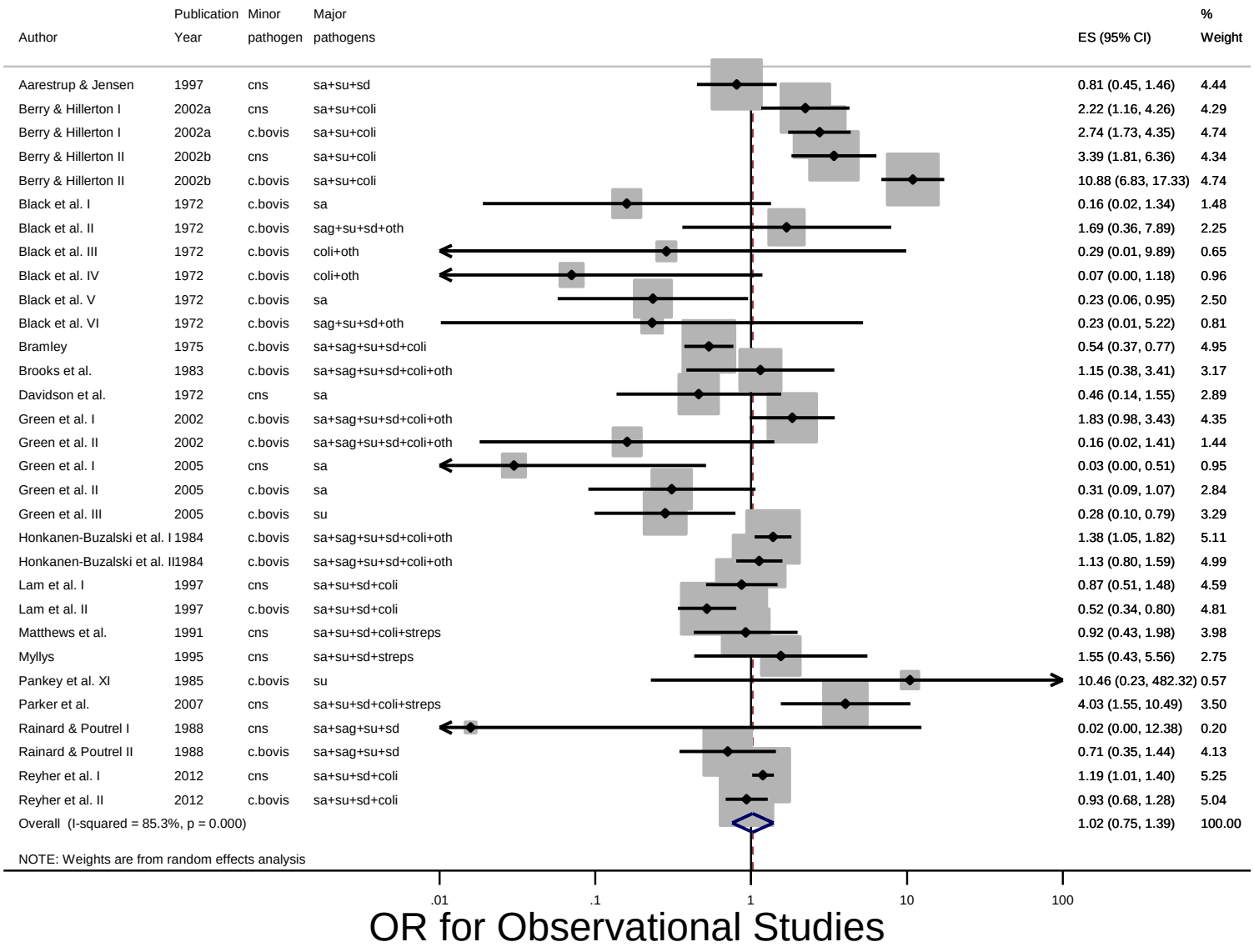

Author
Publication Year
Minor pathogen
Major pathogens
ES (95% CI)
% Weight
Aarestrup & Jensen 1997 cns sa+su+sd 0.81 (0.45, 1.46) 4.44
Berry & Hillerton I 2002a cns sa+su+coli 2.22 (1.16, 4.26) 4.29
Berry & Hillerton I 2002a c.bovis sa+su+coli 2.74 (1.73, 4.35) 4.74
Berry & Hillerton II 2002b cns sa+su+coli 3.39 (1.81, 6.36) 4.34
Berry & Hillerton II 2002b c.bovis sa+su+coli 10.88 (6.83, 17.33) 4.74
Black et al. I 1972 c.bovis sa 0.16 (0.02, 1.34) 1.48
Black et al. II 1972 c.bovis sag+su+sd+oth 1.69 (0.36, 7.89) 2.25
Black et al. III 1972 c.bovis coli+oth 0.29 (0.01, 9.89) 0.65
Black et al. IV 1972 c.bovis coli+oth 0.07 (0.00, 1.18) 0.96
Black et al. V 1972 c.bovis sa 0.23 (0.06, 0.95) 2.50
Black et al. VI 1972 c.bovis sag+su+sd+oth 0.23 (0.01, 5.22) 0.81
Bramley 1975 c.bovis sa+sag+su+sd+coli 0.54 (0.37, 0.77) 4.95
Brooks et al. 1983 c.bovis sa+sag+su+sd+coli+oth 1.15 (0.38, 3.41) 3.17
Davidson et al. 1972 cns sa 0.46 (0.14, 1.55) 2.89
Green et al. I 2002 c.bovis sa+sag+su+sd+coli+oth 1.83 (0.98, 3.43) 4.35
Green et al. II 2002 c.bovis sa+sag+su+sd+coli+oth 0.16 (0.02, 1.41) 1.44
Green et al. I 2005 cns sa 0.03 (0.00, 0.51) 0.95
Green et al. II 2005 c.bovis sa 0.31 (0.09, 1.07) 2.84
Green et al. III 2005 c.bovis su 0.28 (0.10, 0.79) 3.29
Honkanen-Buzalski et al. I 1984 c.bovis sa+sag+su+sd+coli+oth 1.38 (1.05, 1.82) 5.11
Honkanen-Buzalski et al. II 1984 c.bovis sa+sag+su+sd+coli+oth 1.13 (0.80, 1.59) 4.99
Lam et al. I 1997 cns sa+su+sd+coli 0.87 (0.51, 1.48) 4.59
Lam et al. II 1997 c.bovis sa+su+sd+coli 0.52 (0.34, 0.80) 4.81
Matthews et al. 1991 cns sa+su+sd+coli+streps 0.92 (0.43, 1.98) 3.98
Myllys 1995 cns sa+su+sd+streps 1.55 (0.43, 5.56) 2.75
Pankey et al. XI 1985 c.bovis su 10.46 (0.23, 482.32) 0.57
Parker et al. 2007 cns sa+su+sd+coli+streps 4.03 (1.55, 10.49) 3.50
Rainard & Poutrel I 1988 cns sa+sag+su+sd 0.02 (0.00, 12.38) 0.20
Rainard & Poutrel II 1988 c.bovis sa+sag+su+sd 0.71 (0.35, 1.44) 4.13
Reyher et al. I 2012 cns sa+su+sd+coli 1.19 (1.01, 1.40) 5.25
Reyher et al. II 2012 c.bovis sa+su+sd+coli 0.93 (0.68, 1.28) 5.04
Overall (I-squared = 85.3%, p = 0.000) 1.02 (0.75, 1.39) 100.00
NOTE: Weights are from random effects analysis
.01
.1
1
10
100
OR for Observational Studies

Figure 3.

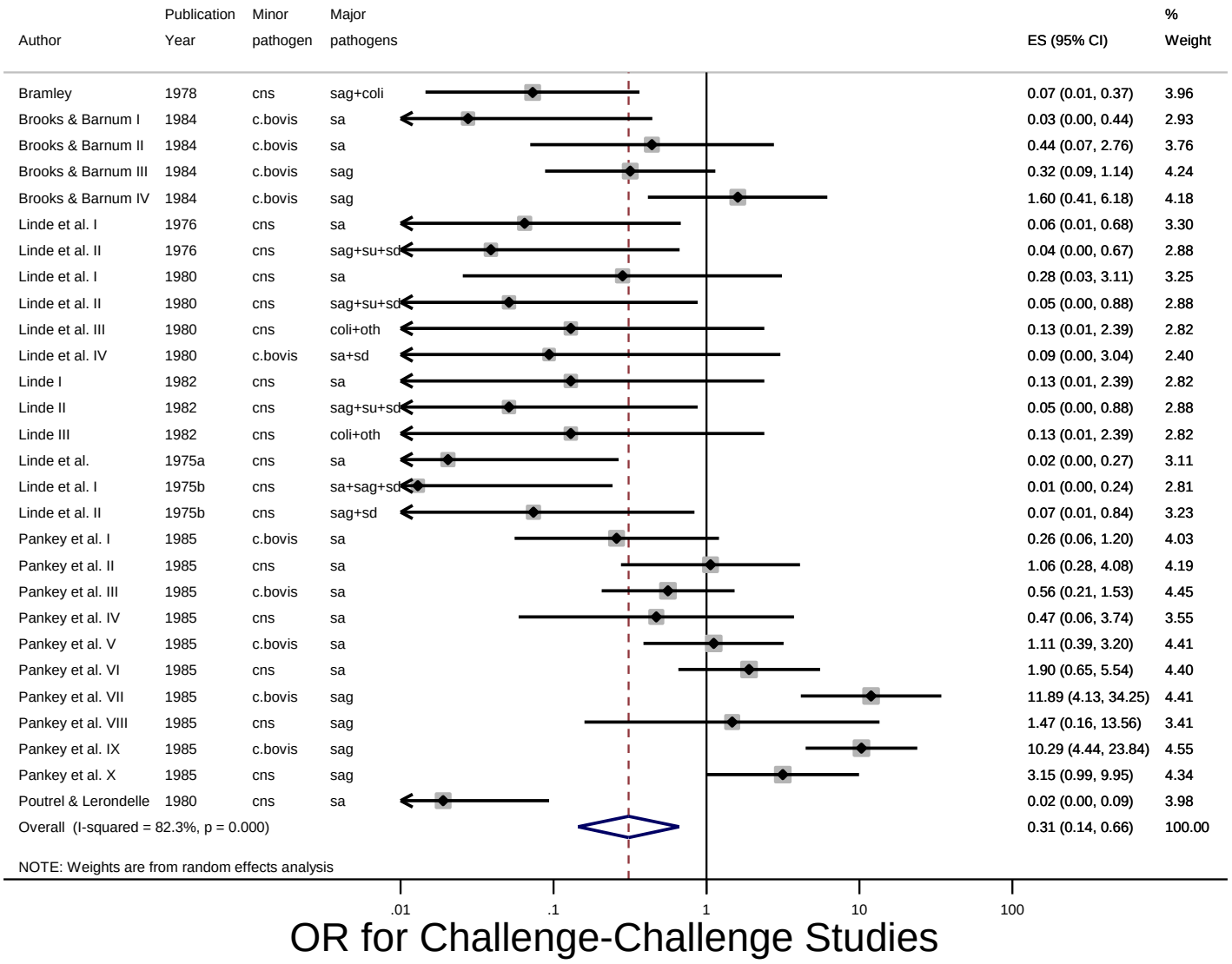

Author
Publication Year
Minor pathogen
Major pathogens
ES (95% CI)
% Weight
Bramley 1978 cns sag+coli 0.07 (0.01, 0.37) 3.96
Brooks & Barnum I 1984 c.bovis sa 0.03 (0.00, 0.44) 2.93
Brooks & Barnum II 1984 c.bovis sa 0.44 (0.07, 2.76) 3.76
Brooks & Barnum III 1984 c.bovis sag 0.32 (0.09, 1.14) 4.24
Brooks & Barnum IV 1984 c.bovis sag 1.60 (0.41, 6.18) 4.18
Linde et al. I 1976 cns sa 0.06 (0.01, 0.68) 3.30
Linde et al. II 1976 cns sag+su+sd 0.04 (0.00, 0.67) 2.88
Linde et al. I 1980 cns sa 0.28 (0.03, 3.11) 3.25
Linde et al. II 1980 cns sag+su+sd 0.05 (0.00, 0.88) 2.88
Linde et al. III 1980 cns coli+oth 0.13 (0.01, 2.39) 2.82
Linde et al. IV 1980 c.bovis sa+sd 0.09 (0.00, 3.04) 2.40
Linde I 1982 cns sa 0.13 (0.01, 2.39) 2.82
Linde II 1982 cns sag+su+sd 0.05 (0.00, 0.88) 2.88
Linde III 1982 cns coli+oth 0.13 (0.01, 2.39) 2.82
Linde et al. 1975a cns sa 0.02 (0.00, 0.27) 3.11
Linde et al. I 1975b cns sa+sag+sd 0.01 (0.00, 0.24) 2.81
Linde et al. II 1975b cns sag+sd 0.07 (0.01, 0.84) 3.23
Pankey et al. I 1985 c.bovis sa 0.26 (0.06, 1.20) 4.03
Pankey et al. II 1985 cns sa 1.06 (0.28, 4.08) 4.19
Pankey et al. III 1985 c.bovis sa 0.56 (0.21, 1.53) 4.45
Pankey et al. IV 1985 cns sa 0.47 (0.06, 3.74) 3.55
Pankey et al. V 1985 c.bovis sa 1.11 (0.39, 3.20) 4.41
Pankey et al. VI 1985 cns sa 1.90 (0.65, 5.54) 4.40
Pankey et al. VII 1985 c.bovis sag 11.89 (4.13, 34.25) 4.41
Pankey et al. VIII 1985 cns sag 1.47 (0.16, 13.56) 3.41
Pankey et al. IX 1985 c.bovis sag 10.29 (4.44, 23.84) 4.55
Pankey et al. X 1985 cns sag 3.15 (0.99, 9.95) 4.34
Poutrel & Lerondelle 1980 cns sa 0.02 (0.00, 0.09) 3.98
Overall (I-squared = 82.3%, p = 0.000) 0.31 (0.14, 0.66) 100.00
NOTE: Weights are from random effects analysis
.01
.1
1
10
100
OR for Challenge-Challenge Studies

Figure 4.

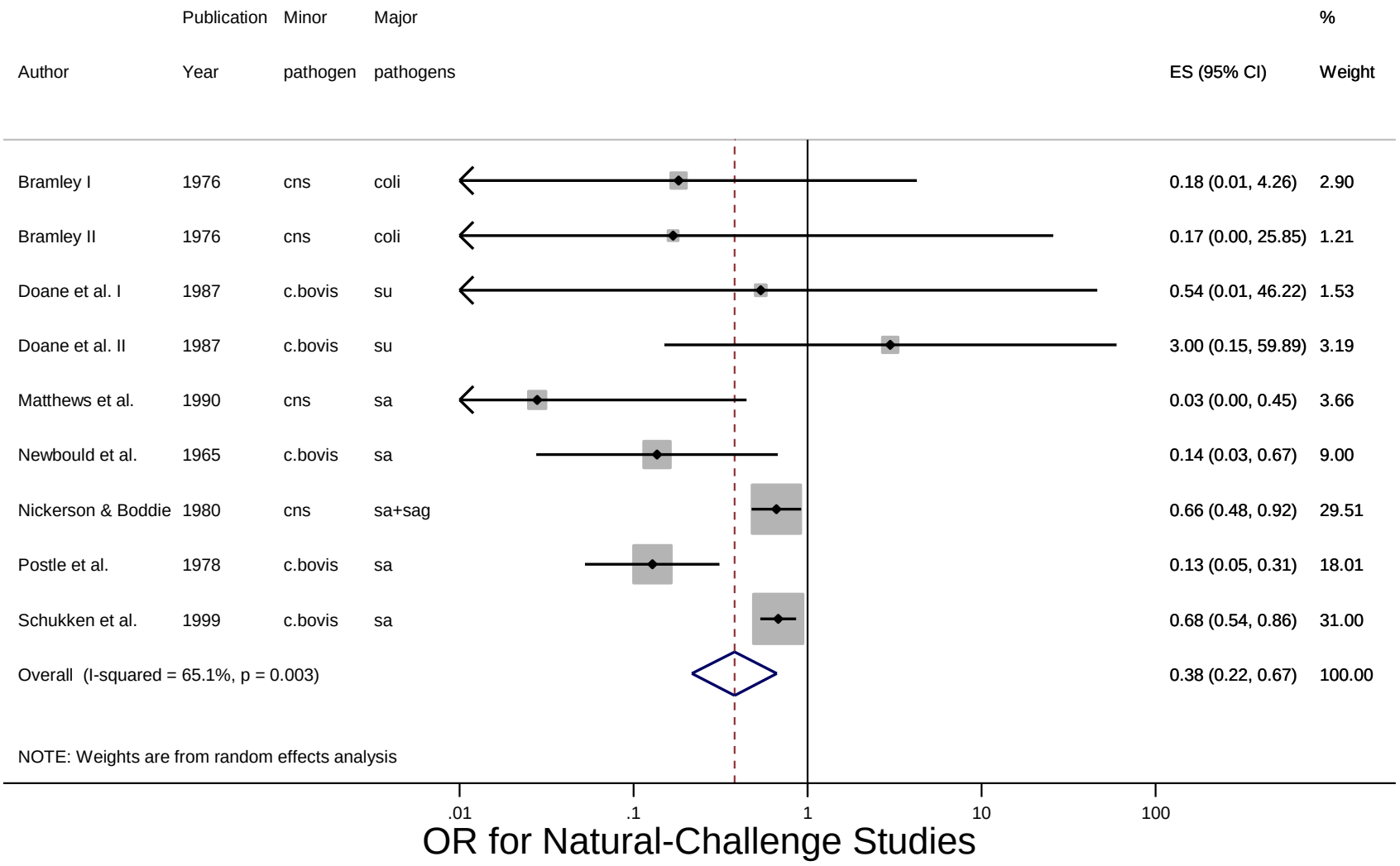
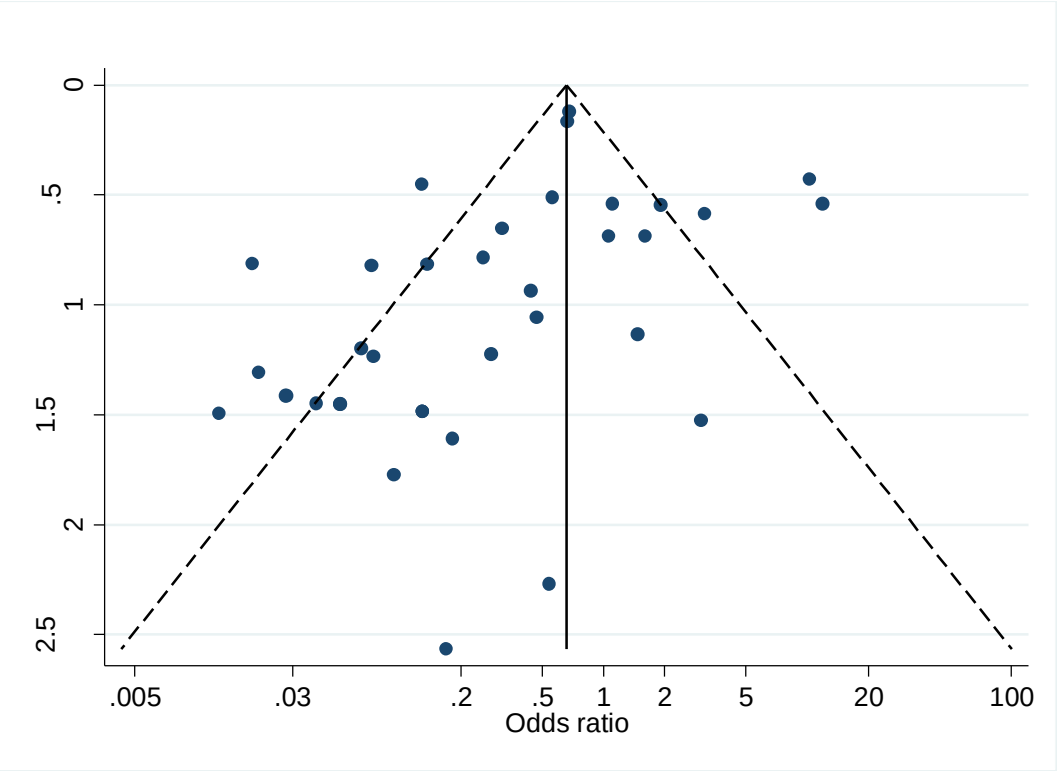

Author
Publication Year
Minor pathogen
Major pathogens
ES (95% CI)
%
Weight
Bramley I 1976 cns coli 0.18 (0.01, 4.26) 2.90
Bramley II 1976 cns coli 0.17 (0.00, 25.85) 1.21
Doane et al. I 1987 c.bovis su 0.54 (0.01, 46.22) 1.53
Doane et al. II 1987 c.bovis su 3.00 (0.15, 59.89) 3.19
Matthews et al. 1990 cns sa 0.03 (0.00, 0.45) 3.66
Newbould et al. 1965 c.bovis sa 0.14 (0.03, 0.67) 9.00
Nickerson & Boddie 1980 cns sa+sag 0.66 (0.48, 0.92) 29.51
Postle et al. 1978 c.bovis sa 0.13 (0.05, 0.31) 18.01
Schukken et al. 1999 c.bovis sa 0.68 (0.54, 0.86) 31.00
Overall (I-squared = 65.1%, p = 0.003) 0.38 (0.22, 0.67) 100.00
NOTE: Weights are from random effects analysis
.01
.1
1
10
100
OR for Natural-Challenge Studies

Figure 5.



Appendix 1. Overview and descriptive information of 38 manuscripts comprising 68 studies used in meta-analyses examining the effect of minor pathogen intramammary infection on new intramammary infections with major pathogens

Reference (year)	Study design[1]	Min. path.[2]	Major pathogens[3]	n[4]	Parity[5]	Stage[6]
Aarestrup and Jensen (1997)	O	CNS	SA, SU, SD	180	H	lact
Berry and Hillerton (2002a) I[7]	O	CNS	SA, SU, coli	290	nd	dry
Berry and Hillerton (2002a) II	O	CB	SA, SU, coli	290	nd	dry
Berry and Hillerton (2002b) I	O	CNS	SA, SU, coli	401	nd	dry
Berry and Hillerton (2002b) II	O	CB	SA, SU, coli	401	nd	dry
Black et al. (1972) I	O	CB	SA	32	nd	lact
Black et al. (1972) II	O	CB	Sag, SU, SD, oth	32	nd	lact
Black et al. (1972) III	O	CB	coli, oth	32	nd	lact
Black et al. (1972) IV	O	CB	coli, oth	38	nd	lact
Black et al. (1972) V	O	CB	SA	38	nd	lact
Black et al. (1972) VI	O	CB	SAg, SU, SD, oth	38	nd	lact
Bramley (1975)	O	CB	SA, SAg, SU, SD, coli	nd[8]	nd	both
Bramley (1976) I	NC	CNS	coli	6	H+L	lact
Bramley (1976) II	NC	CNS	coli	4	H+L	dry
Bramley (1978)	CC	CNS	SAg, coli	17	nd	lact
Brooks et al. (1983)	O	CB	SA, SAg, SU, SD, coli, oth	nd	H+L	both
Brooks and Barnum (1984) I	CC	CB	SA	32	nd	lact
Brooks and Barnum (1984) II	CC	CB	SA	32	nd	lact
Brooks and Barnum (1984) III	CC	CB	SAg	32	nd	lact
Brooks and Barnum (1984) IV	CC	CB	SAg	32	nd	lact
Davidson et al. (1992)	O	CNS	SA	84	L	lact
Doane et al. (1987) I	NC	CB	SU	18	nd	lact
Doane et al. (1987) II	NC	CB	SU	4	nd	lact
Green et al. (2002) I	O	CB	SA, SAg, SU, SD, coli, oth	480	nd	both
Green et al. (2002) II	O	CB	SA, SAg, SU, SD, coli, oth	480	nd	both
Green et al. (2005) I	O	CNS	SA	480	nd	dry
Green et al. (2005) II	O	CB	SA	480	nd	dry
Green et al. (2005) III	O	CB	SU	480	nd	dry
Honkanen-Buzalski et al. (1984) I	O	CB	SA, SAg, SU, SD, coli, oth	1450	nd	both
Honkanen-Buzalski et al. (1984) II	O	CB	SA, SAg, SU, SD, coli, oth	1450	nd	both
Lam et al. (1997) I	O	CNS	SA, SU, SD, coli	392	nd	both
Lam et al. (1997) II	O	CB	SA, SU, SD, coli	392	nd	both
Linde et al. (1975a)	CC	CNS	SA	9	nd	lact
Linde et al. (1975b) I	CC	CNS	SA, SAg, SD	14	nd	lact
Linde et al. (1975b) II	CC	CNS	SAg, SD	5	nd	lact
Linde et al. (1976) I	CC	CNS	SA	6	nd	lact
Linde et al. (1976) II	CC	CNS	SAg, SU, SD	7	nd	lact
Linde et al. (1980) I	CC	CNS	SA	8	nd	lact
Linde et al. (1980) II	CC	CNS	SAg, SU, SD	7	nd	lact
Linde et al. (1980) III	CC	CNS	Coli, oth	9	nd	lact
Linde et al. (1980) IV	CC	CB	SA, SD	2	nd	lact
Linde (1982) I	CC	CNS	SA	8	nd	lact
Linde (1982) II	CC	CNS	SAg, SU, SD	7	nd	lact
Linde (1982) III	CC	CNS	Coli, oth	9	nd	lact
Matthews et al. (1990)	NC	CNS	SA	10	nd	lact
Matthews et al. (1991)	O	CNS	SA, SU, SD, coli, oth	113	H+L	both
Myllys (1995)	O	CNS	SA, SU, SD, oth	50	H	dry
Newbould et al. (1965)	NC	CB	SA	10	H+L	lact

Nickerson and Boddie (1994)	NC	CNS	SA, SAg	600	nd	lact
Pankey et al. (1985) I	CC	CB	SA	57	nd	lact
Pankey et al. (1985) II	CC	CNS	SA	57	nd	lact
Pankey et al. (1985) III	CC	CB	SA	nd	nd	lact
Pankey et al. (1985) IV	CC	CNS	SA	nd	nd	lact
Pankey et al. (1985) V	CC	CB	SA	nd	nd	lact
Pankey et al. (1985) VI	CC	CNS	SA	nd	nd	lact
Pankey et al. (1985) VII	CC	CB	SAg	nd	nd	lact
Pankey et al. (1985) VIII	CC	CNS	SAg	nd	nd	lact
Pankey et al. (1985) IX	CC	CB	SAg	nd	nd	lact
Pankey et al. (1985) X	CC	CNS	SAg	nd	nd	lact
Pankey et al. (1985) XI	O	CB	SU	nd	nd	dry
Parker et al. (2007)	O	CNS	SA, SU, SD, coli, oth	255	H	dry
Postle et al. (1978)	NC	CB	SA	41	H+L	lact
Poutrel and Lerondelle (1980)	CC	CNS	SA	44	H	lact
Rainard and Poutrel (1988) I	O	CNS	SA, SAg, SU, SD	122	H+L	lact
Rainard and Poutrel (1988) II	O	CB	SA, SAg, SU, SD	122	H+L	lact
Reyher et al. (2012) I	O	CNS	SA, SU, SD, coli	6825	H+L	lact
Reyher et al. (2012) II	O	CB	SA, SU, SD, coli	6825	H+L	lact
Schukken et al. (1999)	NC	CB	SA	145	H+L	lact

[1]Study design: observational (O), challenge studies where quarters were experimentally challenged both with a minor pathogen and subsequently with a major pathogen (CC), challenge studies where quarters were naturally infected with a minor pathogen and challenged with a major pathogen (NC)

[2]Minor pathogens represented: coagulase-negative staphylococci (CNS), *Corynebacterium bovis* (CB)

[3]Major pathogens represented: *Staphylococcus aureus* (SA), *Streptococcus agalactiae* (SAg), *Streptococcus uberis* (SU), *Streptococcus dysgalactiae* (SD), coliforms (coli), other streptococci, *Pseudomonas* spp., yeast, etc. (oth)

[4]Number of cows

[5]Parity: Heifers only (H), lactating cows only (L), heifers and lactating cows (H+L), not described (nd)

[6]Stage of lactation: Lactation only (lact), dry period only (dry), both lactation and dry period (both)

[7]Study number from a single reference

[8]Not described (nd)