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Methodologic quality and genotyping reproducibility in studies of tumor necrosis factor –308 G→A single nucleotide polymorphism and bacterial sepsis: Implications for studies of complex traits*

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Abstract

Objective—Studies of genetic associations with common diseases, such as between cytokine gene polymorphisms and severe bacterial sepsis, have reached conflicting conclusions. Failure to follow methodologic standards may have contributed to discordant findings. The –308 G→A transition in the tumor necrosis factor– promoter has been genotyped by a variety of methods. Based on our observation of genotyping inaccuracies, we sought to determine whether published studies followed a series of acceptable methodologic standards and whether failure to follow the standard of genotyping reproducibility could lead to erroneous conclusions about gene-disease associations.

Design—Systematic review and reanalysis of banked genetic material. We applied a published series of seven methodologic standards to five reports of the association between this variant and bacterial sepsis. We then studied the accuracy of restriction fragment length polymorphism for the –308 site using DNA from a cohort of injury victims.

Setting—Surgery research laboratory.

Measurements and Main Results—We observed that methodologic quality was not uniform and that reproducibility of genotyping was infrequently met. In our subjects, we found that 4 of 46 heterozygotes analyzed by restriction fragment length polymorphism were actually GG-homozygotes (9% misclassified) according to alternative genotyping methods.

Conclusions—Failure to confirm genotype may have led to conclusions that this polymorphism is not associated with sepsis or outcome. Our observations have implications for the conduct and evaluation of studies of complex genetic disease.

Keywords

bacterial sepsis; genotyping; tumor necrosis factor- α

It has been observed that a substantial fraction of studies examining associations between genetic differences and clinical conditions fall short on a number of basic methodologic standards (1). A series of such standards has been proposed for studies examining gene polymorphisms as risk factors for disease and, although not necessarily representing a broad consensus, are based on established epidemiologic principles (Table 1, adapted from reference

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Bogardus et al (1)). One such potential genotype-phenotype association is that between the single nucleotide polymorphism (SNP) at the -308 position in the tumor necrosis factor (TNF)- α gene promoter (G→A transition) and the risk for and outcome from severe bacterial infection (severe sepsis, septic shock, and death) (2). Discordant results have been attributed to the polymorphism having no functional effect, to inadequate sample sizes, and to linkage disequilibrium between the -308 variant and other (potentially unidentified) functional loci. However, such discordance may arise, at least in part, from methodologic inadequacies such as incorrect genotype assignment. Herein, we report our observations of this potential inadequacy and how it may have had an impact of the conclusions in published reports.

MATERIALS AND METHODS

Source of Genetic Material

With approval from the Institutional Review Board at the University of Texas Southwestern Medical Center, a blood sample was drawn from patients with severe injury (blunt, penetrating, or burn trauma) who provided informed consent. Genomic DNA was extracted from ethylenediaminetetraacetic acid anticoagulated blood, using Qiagen's QIAamp DNA Blood Midi Kit (Valencia, CA) according to manufacturer's instructions and stored at -20°C at a concentration of 100 ng/ μ L.

Restriction Fragment Length Polymorphism Detection

A 107 base pair fragment encompassing the TNF -308 position was amplified as described by Wilson et al. (3,4); 15 μ L of polymerase chain reaction (PCR) product was incubated at 37°C for 2 hrs with 5 units of *NcoI* restriction enzyme (Promega, Madison, WI), 0.1 mg/mL bovine serum albumin, and 1 $\times$ buffer containing 60 mM Tris-HCl, (pH 7.9), 1.5 M NaCl, 60 mM MgCl₂, and 10 mM dithiothreitol in a total volume of 25 μ L; 10 $\times$ stop solution (20% Ficoll, 1% sodium dodecylsulfate, 0.25% bromophenol blue, 75 mM ethylenediaminetetraacetic acid) was added at a concentration of 5% total volume after restriction. Fragments were electrophoresed on a 3% Nu Sieve agarose gel (Bio-Whittaker Molecular Applications, Rockland, ME), stained with 0.5 μ g/mL ethidium bromide, and photographed. Unrestricted PCR products were run alongside the restricted sample for comparison. The presence of a single band (107 base pairs) represents a GG-homozygote, two bands (87 base pairs and 20 base pairs) indicate an AA-homozygote, and three bands (107 base pairs, 87 base pairs, and 20 base pairs) indicate a heterozygote. One individual (D. L. Peters) was responsible for assigning genotype by restriction digestion. Of the three genotyping methods employed, restriction fragment length polymorphism (RFLP) is the most highly subjective, being based on visual identification of patterns on a gel. Therefore, the data from this method were recorded before the other methods, and we minimized potential inaccuracies by confirming all determinations with a second review (G. E. O'Keefe).

Dye Terminator Sequencing

A 511 base pair fragment extending from -501 to +11, encompassing the -308 SNP, was amplified by PCR (primers and conditions shown in Table 1) and then purified using Qiaquick Multiwell PCR Purification (Qiagen) according to the manufacturer's instructions. Twenty-five femtomoles of purified product was cycle sequenced using the CEQ DTCS Dye Terminator Cycle Sequencing Kit (Beckman Coulter, Fullerton, CA) using the reverse PCR primer; 5% dimethyl sulfoxide and 1.5 mM MgCl₂ were added to each reaction. Reactions were cycled 50 times at 96°C for 30 secs, 50°C for 30 secs, and 60°C for 4 mins. Samples were sequenced on a Beckman CEQ 2000XL sequencer and analyzed for heterozygosity via software analysis and visual inspection. Genotype assignment by sequencing was done by one individual (E. M. Flood) and recorded in the database by another (D. L. Peters).

Pyrosequencing

Pyrosequencing is a technique for rapid polymorphism analysis (5). It is a single base extension method employing streptavidin-coated magnetic beads to capture a single stranded biotinylated PCR fragment. The incorporation of sequentially dispensed nucleotides will cause pyrophosphate to be released and begin a cascade of enzymatic events that result in the generation of light. The light intensity, which is proportional to the number of nucleotides incorporated into the synthesizing strand, is then converted into a readable peak on a pyrogram.

PCR was performed as for the 107 base pair fragment, amplified for restriction digestion except that a 5' biotinylated forward primer replaced the original forward primer, and the SNP sequence was read in the reverse direction. Biotinylated PCR products were attached to streptavidin-coated magnetic beads by incubating and shaking 25 µL of PCR product with 25 µL of 2× binding wash buffer, pH 7.6 (10 mM Tris-HCl, 2 M NaCl, 1 mM ethylenediaminetetraacetic acid, 0.1% Tween 20) and 80 µg of Dynabeads (Dynal AS, Oslo, Norway) at 65°C for 15 mins at 1400 rpm. Using the magnetic PSQ96 sample prep tool, the PCR products were separated from the nonbiotinylated strands by incubation in 50 µL of 0.5M NaOH for 1 min and then washed in 100 µL of 1× annealing buffer, pH 7.6 (20 mM Tris-Acetate, 5 mM magnesium acetatetetrahydrate). The biotinylated strands were annealed to 10 pmol of sequencing primer designed to hybridize to the base immediately adjacent to the SNP (conditions and primers are shown in Table 2) in 1× annealing buffer (total volume, 45 µL) at 80°C for 2 mins. Samples were allowed to cool to room temperature and then placed in the PSQ96. Samples were analyzed using pattern recognition software and recorded by one individual (D. L. Peters). Like sequencing, pyrosequencing is a highly objective method; however, the reactions infrequently fail or the computer does not assign a genotype with certainty. In these cases, one author (G. E. O'Keefe) reviewed all unclear assignments, and if not resolved, the sample was subjected to repeat pyrosequencing.

Selection of Published Reports

We followed the outline described in the *Users' Guides to the Medical Literature* for the process of conducting a systematic review. Although it was not our intention to pool and analyze the data as a formal meta-analysis, we did follow the guidelines for defining the question, conducting the literature search, applying inclusion/exclusion criteria, and abstracting the data from the studies. Specifically, we reviewed the literature for studies that evaluated the association of the -308 polymorphism and the risk for and mortality from severe bacterial sepsis. We searched MEDLINE (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi>) for articles containing the terms: TNF and polymorphism and sepsis. The resulting 28 articles were reviewed, and only those that reported original data regarding the effect of this SNP on the end points of complicated sepsis (severe sepsis or septic shock) or mortality from sepsis were included. We also reviewed the references listed in each of these for additional original articles that were not identified by the initial search. This search identified five original articles (6–10). Four of these five concerned adult patients with severe sepsis or septic shock. The fifth concerned children with meningococcal infections and was excluded (6). Through peer discussion, we identified one additional study reporting on the association between this polymorphism and severe sepsis (11).

Characterization of Methodologic Quality of Individual Studies

Three of the co-authors (R. C. Barber, D. L. Peters, and G. E. O'Keefe) independently applied the previously published criteria and definitions to these five studies. Unlike Bogardus et al. (1), we did not exclude smaller studies, such as those with less than ten case subjects for each allele. However, by all other measures, we followed their reported approach.

RESULTS

Comparison of Genotyping Methods

We analyzed samples from 291 subjects for the polymorphism using at least two methods. All subjects were admitted to the intensive care unit at Parkland Memorial Hospital for the treatment of severe traumatic injuries ($n = 174$) or major burns ($n = 117$). All were genotyped by restriction digestion and pyrosequencing, and 100 were genotyped by dye terminator sequencing. These 100 included all 48 A-allele carriers (2 AA-homozygotes and 46 heterozygotes) and 52 randomly selected GG-homozygotes (based on RFLP). A subgroup of these patients is the subject of a recent publication examining the association between three TNF α promoter polymorphisms and severe sepsis after traumatic injury (12). There was disagreement between RFLP and sequencing in four cases. Each was classified as a heterozygote by RFLP but classified as GG-homozygotes by sequencing. Repeat sequencing confirmed GG-homozygosity in each case, and a more prolonged period of digestion (24 hrs) did not resolve the discordance between RFLP restriction digestion and sequencing. Therefore, 4 of 46 heterozygotes (9%; 95% confidence interval, 1–16%) identified by RFLP were misclassified GG-homozygotes when dye terminator sequencing was considered the gold standard. These results are summarized in Table 3. In all 100 cases, pyrosequencing concurred with the results of dye terminator sequencing. For the remaining samples that were classified as GG-homozygous by RFLP (and not analyzed by dye terminator sequencing), pyrosequencing corresponded with the results of restriction digestion.

Studies Examining the Association Between –308 A-Allele Carriage, Complicated Sepsis, and Death

All five studies reported on the association between mortality, and three reported an association between the severity of sepsis and genotype. By independent review, all three reviewers reached the same determination of whether a study met each criterion. A summary of these assessments is shown in Tables 4 and 5. Study 1 satisfied all seven criteria and study 2 met only three criteria. The remaining studies met an intermediate number. For all studies, the case and comparison groups were defined with sufficient detail (third and fifth criteria). Although most studies had few subjects, the criteria for case and comparison group adequacy (fourth and sixth criteria) were met, with one exception. In study 2, the allele frequency in 80 patients with severe sepsis was compared with the allele frequencies in a younger, healthy, control group. Moreover, in a second comparison of allele frequencies between patients with severe sepsis who lived and those who died, there was no indication of the distribution of other known mortality risk factors (such as age and presence of shock) in the two groups. Thus, the standards for adequacy of cases and comparison groups were not met. All studies met the criterion for quantification of results (seventh criterion). Study 1 and study 4 were the only two indicating that the individual assigning genotype did so blinded to the clinical outcome information and therefore were the only studies to satisfy the objectivity standard (second criterion). Reproducibility (first criterion) was the methodologic standard least often satisfied; only study 1 indicated that variant alleles were confirmed by an additional genotyping method. None of the other four studies indicated that any of the genotype assignments were confirmed by repeat analysis by the same or an additional technique.

DISCUSSION

The sequencing of the human genome, the ability to genotype large groups of subjects, and the computational capacity to deal with the data generated have facilitated the exponential increase in the studies exploring the genetic basis of common diseases (2). Such advances do not negate (perhaps they amplify) the importance of following traditional epidemiologic principles in the conduct and analysis of these studies. There is considerable controversy regarding the effects

of and associations with the TNF α -308 SNP (13). We have observed that published reports examining the association between this polymorphism and bacterial sepsis in adults vary in a number of indicators of methodologic quality. Moreover, the discordant findings of these studies may be the consequence of methodologic inadequacies, an explanation for which may lie in the almost universal failure to meet one of the suggested standards: reproducibility. We cannot say that genotype misclassification was responsible for the lack of association observed in the articles reviewed. The only definitive way to do so would be to reexamine the genetic material from those reports. Unfortunately, the authors of the studies that we have identified and were able to contact were not able to provide us with genetic material to test our hypothesis. We are thus left without definitive information regarding the accuracy of the genotyping methods on which these publications are based. However, if the studies relying solely on a single genotyping method falsely identified 9% of individuals as heterozygotes, the interpretation could be materially different if the correct genotype was assigned. If we assume that one of ten heterozygotes was actually a GG-homozygote and assume that patients with good outcomes (survived or did not develop septic shock) were those who were misclassified, the interpretation of the three studies would change considerably. For instance, Tang et al. (9) identified a nonstatistically significant increased risk for septic shock associated with A-allele carriage in their patients with surgical infections (relative risk, 1.48; $p = .13$). If three of the 26 classified as A-allele (~10%) carriers were truly GG-homozygotes, and the three that were misclassified did not develop shock, the relative risk would have been reported as 1.7 ($p = .03$) and likely interpreted differently. A similar change in the interpretation of mortality data would also be evident. A similar percentage change in genotype assignment in the study of Stuber et al. (10) would change the relative risk for severe sepsis from 1.0 ($p = .9$) to 1.3 ($p = .16$). Although, in this case, not a “statistically significant” association, the implication of genotype misclassification is clear. In these hypothetical but reasonable modifications, we applied the error rate that we observed (~10%) and assumed that all misclassified individuals did not develop the adverse outcome of interest. We do not propose that this indeed occurred in the studies reviewed but simply suggest that this possibility may have lead to inaccuracy in the published reports. A lower misclassification rate or a circumstance in which both outcomes were represented in the misclassified individuals would result in less marked changes in the risk estimates.

Although the study meeting all of the methodologic standards identified an increased risk for septic shock and death from septic shock in carriers of the -308 A-allele, we did not intend to determine, nor do we conclude from our observations here, that there is an association between this polymorphism and severe bacterial infection in adults (7). Our intention was more general: specifically, that genotype misclassification may occur, particularly when relying on a single method, and may result in bias sufficient to result in incorrect conclusions. The particular genotyping method used in these five reports warrants discussion given our observations regarding potential genotyping inaccuracies. Studies 2, 4, and 5 reported that the risk for septic shock and that mortality from septic shock were not related to the A-allele. Each of these studies relied solely on genotyping by RFLP, the method that we observed to misclassify 4 of 46 heterozygotes.

If the studies relying solely on a single genotyping method falsely identified 9% of individuals as heterozygotes, the interpretation could be materially different if the correct genotype was assigned.

There are a number of explanations for our observation of genotype misclassification. Preferential amplification of one DNA strand over another in heterozygotes may be due to mismatches between the primer and the allelic template, altered melting profiles, and hence, efficiency of cycling during the PCR and allelic exclusion due to alterations in the storage conditions (14–16). However, this is unlikely to explain our findings in which homozygotes were misclassified as heterozygotes. Of particular consideration here is that this SNP does not

generate a natural restriction site for any endonuclease; therefore, it is necessary to create one by incorporating a base mismatch into the PCR product. For this SNP, an *NcoI* restriction site is created by a mismatched base (cytosine at -303) (17). It is possible that this restriction site may not be created due to preferential amplification of the native strand (which, after 30 PCR cycles, would normally represent $\ll 1\%$ of the PCR product). This could lead to a substantial fraction of DNA fragments not having the forced restriction site, despite having guanine at -308. Digestion of DNA fragment would result in three bands that are otherwise characteristic of the heterozygous state. The alternative explanation of incomplete restriction was theoretically possible but would not negate the importance of our findings as the published reports used a similar duration of digestion (2–4 hrs). Unlike in the case of the TNF α -308 polymorphism, many SNPs do introduce a natural restriction site, and the accuracy of their detection may not be reduced in the manner described above as the efficiency of amplification between the variant sequences are likely not to be different. It is thus possible that our observations are applicable only to SNPs that do not result in a natural restriction site. However, we have used both RFLP and pyrosequencing (but not dye terminator sequencing) to examine SNPs in other genes (interleukin-1 β , interleukin-6, CD14, and TNF β) that do result in natural restriction sites. Our observations were that RFLP and pyrosequencing corresponded in all cases for the interleukin-1 β , interleukin-6, and CD14 SNPs but not for the TNF β SNP. Of 134 samples genotyped for the TNF β by both RFLP and pyrosequencing, the two methods did not agree 22 times (16%). Therefore, although we do not extrapolate our observations to other polymorphisms, particularly those interrogated by methods other than RFLP, we provide empirical evidence that not satisfying the reproducibility standard can result in null findings. It is possible that RFLP is least accurate under the circumstances in which the restriction site is forced, although it seems that even in the case of natural restriction sites, that RFLP may misclassify individuals.

RFLP was the genotyping method observed here to result in misclassification. This is a method that has and will continue to be supplanted by newer, more automated, and more rapid techniques. Nonetheless, this method does not require complex equipment and can be easily performed in most molecular biology laboratories. RFLP remains a genotyping technique that continues to be used in moderate to large-scale studies of genotype-phenotype associations (18–20). Furthermore, as the misclassification may be related to preferential amplification during PCR, other genotyping methods that rely on PCR amplification may be subject to this potential bias (14). With recent advances in the ability to perform high-throughput and large-scale genotyping, it is apparent that reproducibility of genotyping takes a high profile during the design of these studies.

Of note is that the overall accuracy of RFLP when compared with dye terminator sequencing was 96%. However, misclassification, limited to falsely identifying A-allele carriers, led to a positive predictive value for identifying this variant by RFLP of 92%. Therefore, investigators should at least consider the possibility of genotype misclassification as they design studies to examine the association of SNPs and complex diseases such as sepsis. In general, misclassification as identified here will reduce the power of a study to detect risk differences between genotypes and can be, in part, overcome by increasing the number of subjects enrolled. Therefore, a combination of minimizing misclassification (by adhering to the concept of genotyping reproducibility) and enrolling a sufficient number of subjects to overcome the effects of a minimal misclassification rate will be necessary aspects of future studies examining genetic influences on the risk for and outcome from critical illnesses.

It is possible that factors unrelated to these seven methodologic standards are responsible for the discordant published findings. For instance, a single SNP may not reflect unique interactions that might exist among genetically linked loci, or haplotypes, such as those that occur in the interleukin-6 and $\beta 2$ receptor genes (21,22). In each of these genes, there are

differences in *in vitro* responses that are related to haplotype and not necessarily individual SNPs. Another important factor that may have contributed to the discordant findings is difference in the genetic background (23). Such differences may mask real associations between genes and the end points of interest. Furthermore, the utility of independent confirmation of genotype-phenotype associations has recently been questioned, but no satisfactory alternative has been devised (24).

CONCLUSIONS

In summary, we recommend that authors follow the set of guidelines proposed by Bogardus et al. when conducting studies examining associations among genetic variants and risk for and outcomes from complex diseases. Furthermore, journal editors and reviewers should consider these criteria when reviewing manuscripts describing data that attempt to characterize genetic associations with complex conditions such as severe sepsis.

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Table 1

Definitions of methodologic standards

Standard	Definition
Reproducibility	This refers to whether the genetic variation was confirmed by more than one genotyping method or, alternatively, whether the authors at least mentioned that variant alleles were confirmed by the same method more than once.
Objectivity	This refers specifically to whether genotype and phenotype (in the case here, severe sepsis or septic shock) were each assigned independently (blinded) of the other.
Definition of cases Adequate spectrum of cases	Case description should be in sufficient detail such that other investigators could assemble a similar group. The conclusions reached by the article must be supported by the spectrum of cases studied; this spectrum refers to a number of features, ranging from demographic to range of illness severity.
Definition of comparison group Adequacy of comparison group	As with the case subjects, the comparison (control) group should be described in sufficient detail to be reproduced by others. Healthy subjects from the community from which the case subjects arise are considered acceptable in case-control studies; for cohort studies, the comparison group (at risk for septic shock or death, for example) should be similar to the cases except for the outcome being studied.
Quantification of association	This requires that the main comparison be supported by a quantification of the difference between study groups (percentage of risk in each group or relative risk are each considered acceptable) and a statistical estimate such as a <i>p</i> value or confidence interval.

Table 2

Primer pairs and polymerase chain reaction conditions for genotyping

Genotyping Method	PCR Product Length	Primer Concentrations	Primer Pairs	Cycling Conditions
–308 Restriction digestion	107 base pairs	5 pmol	Forward: –327 AGGCAATAGGTTTGTGAGGGCCAT Reverse: –221 TCCTCCCTGCTCCGATTCCG	95°C for 3 mins/35 cycles of 95°C for 30 secs, 60°C for 30 secs, 74°C for 30 secs/ 74°C for 6 mins
–308 Pyrosequencing	107 base pairs	5 pmol	Forward: –327 Biotin- AGGCAATAGGTTTGTGAGGGGCAT Reverse: –221 TCCTCCCTGCTCCGATTCCG Pyrosequencing primer: R 5'GGCTGAACCCCGTCC	95°C for 3 mins/35 cycles of 95°C for 30 secs, 60°C for 30 secs, 74°C for 30 secs/ 74°C for 6 mins
Sequencing of tumor necrosis factor promoter region (–501 → +11)	511 base pairs	25 pmol	Forward: –496 CAAACACACAGGCTCAGGACT Reverse: +16 CGTCTGCTGGCTGGGTGTGC	95°C for 5 mins/30 cycles of 95°C for 1 min, 61°C for 1 min, 72°C for 1 min/72° C for 7 mins

Table 3

Comparison of restriction fragment length polymorphism (RFLP) and dye terminator sequencing for tumor necrosis factor- α -308 polymorphism

RFLP	Dye Terminator Sequencing		
	GG-Homozygote	A-Allele Carrier	
GG-homozygote	52 (100%)	—	52
A-allele carrier	4 (8%)	44 (92%)	48
	56	44	100

Percentages refer to the proportion of each genotype according to dye terminator sequencing that was correctly classified by RFLP. The A-allele carriers include the heterozygotes and two AA-homozygotes. A total of 8% of A-allele carriers, according to RFLP, were actually GG-homozygotes.

Studies of the tumor necrosis factor (TNF)-α -308 single nucleotide polymorphism (SNP) and bacterial sepsis in adults

Author (citation)	Outcomes Measured	Genotyping method(s)	Summary
Mira et al. (7)	Risk for and mortality from septic shock	1. DGGE	The authors genotyped 89 patients with septic shock and 87 healthy control subjects. Patients with septic shock were more likely to carry the -308 A-allele (39%) than were controls (18%). Patients with septic shock who carried the A-allele were more likely to die.
Stuber et al. (10)	Risk for and mortality from severe sepsis	2. Sequencing 1. RFLP	The authors genotyped 80 patients with severe sepsis and 153 controls for TNF-α -308 and TNF-β +250 SNPs. They found no difference in -308 allele frequencies between cases and controls and no difference between patients with severe sepsis who lived or died.
Appoloni et al. (11)	Mortality from septic shock	1. SSOP	The authors genotyped 34 patients with septic shock. -308 A-allele carriers were more likely to die (78%) than GG-homozygotes (32% died).
Tang et al. (9)	Risk for and mortality from septic shock	1. RFLP	The authors genotyped 112 postoperative patients with infections for the TNF-α -308 SNP. They found no association between genotype and the risk for septic shock or mortality overall. However, A-allele carriers had a higher case-fatality rate than GG-homozygotes when the analysis was limited to patients with septic shock.
Waterer et al. (8)	Risk for septic shock and mortality in patients with CAP	1. RFLP	The authors genotyped 280 patients with CAP for the TNF-α -308 and TNF-β +250 SNPs. They did not observe an association between the development of septic shock (which occurred in 31 patients) or mortality (occurred in 25 patients) and carriage of the A-allele at -308.

DGGE, denaturing gradient gel electrophoresis; RFLP, restriction fragment length polymorphism; SSOP, sequence-specific oligonucleotide priming; CAP, community-acquired pneumonia.

Table 5

Methodologic quality of studies of the tumor necrosis factor- α -308 single nucleotide polymorphism and bacterial sepsis in adults

Methodologic standard	Study 1 (7)	Study 2 (10)	Study 3 (11)	Study 4 (9)	Study 5 (8)
Reproducibility	Yes	No	No	No	No
Definition of cases	Yes	Yes	Yes	Yes	Yes
Adequacy of cases	Yes	No	Yes	Yes	Yes
Definition of comparison group (controls)	Yes	Yes	Yes	Yes	Yes
Adequacy of comparison group	Yes	No	Yes	Yes	Yes
Objectivity	Yes	No	No	No	Yes
Quantification of results	Yes	Yes	Yes	Yes	Yes

Parenthetical number is the reference number for each study.