Forensic microbiology and bite marks: a systematic review

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KEYWORDS

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ABSTRACT

The forensic role of microbiology in bite mark analysis as evidence in a court of law has not yet been explored, as the analysis of bite marks is mostly morphology-based. The aim of this systematic review is to investigate if the analysis of the oral microbiota may be helpful as a complementary forensic tool. Articles were searched on the PubMed database, using predefined data fields and keywords. The final selection included a total of 6 papers (out of 42). Our results indicated that the *Streptococcus* genus is a key player in the analysis of bite mark microbiology from a forensic perspective and its genomic analysis may facilitate the association of a bite mark to the perpetrator. However, much more research is still needed before this forensic strategy can be applied in real scenarios. There is a need to optimize and standardize the methods of microbiome analysis and to determine several factors that may influence the results, such as the frequency of bacterial genotypes in the human population and the temporal stability of the oral microbiome on human skin.

INTRODUCTION

A bite mark can be described as a physical alteration on the skin or other materials caused by teeth pressure.[1] As physical evidence, bite marks are analysed using morphological aspects, and a comparison between the suspect's dentition and the mark inflicted on the victim's skin is performed.[1-6] The analysis of the bite mark as biological evidence may explore the oral DNA left by the biter.[7] This DNA may originate from host cells (human DNA) or from the oral microbiome. Despite being very useful for human identification, human DNA bite mark analysis can be extremely difficult due to DNA's rapid degradation by nucleases present in the saliva or on the skin.[1,2,5,6] In comparison to human DNA, microbiome analysis can offer several advantages, namely due to microbial DNA ubiquity and diversity,[8] greater resistance to degradation (due to their cell wall and biofilm), and to the potential to distinguish monozygotic twins.[9] For these reasons, there is a growing interest in microbiology in forensic science, particularly in human identification. Specifically, oral microbiome may have a great potential in forensic investigation since it presents high diversity and quantity of organisms, high inter-personal variability and intra-personal stability, and also because saliva is an easily accessible biological fluid. Oral microbiota includes more than 700 species of

microorganisms, where the *Streptococcus* is the most prevalent genus, found in saliva and soft tissues.[10] The species *Streptococcus mitis*, *Streptococcus sanguinis,* and *Streptococcus oralis* are the most common initial colonizers of the teeth biofilm.[1]

Forensic microbiology uses microbiological methods in criminal and medico-legal investigations by analysing and interpretating microbial evidence.[1,11] In bite mark microbiology, it is intended to collect and amplify microbial evidence on the victim's skin to associate the biter to the bite mark, and, perhaps, use it as additional evidence in a court of law. To evaluate the viability of the use of microbial DNA as forensic evidence in crimes involving bite marks, we have reviewed experimental trials that addressed the collection of microbial DNA from human bite marks.

This systematic review aims to provide an up-to-date clear and objective assessment of how microbiology can assist the criminal investigation into the perpetuator's identification when a victim presents with a bite mark.

MATERIAL AND METHODS

This review followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) protocol[12] and was registered on the PROSPERO (International Prospective Register of Systematic Review - Centre for Reviews and Dissemination University of York) website with the registration number 2022 CRD42022292232.

The scientific articles chosen for this review were selected from PubMed database between October and December 2021, with its query ((forensic or forensics) AND ("microbiology" or "microbiome" or "*Streptococcus*" or "microorganism" or "microbes" or "microflora" or "microbial" or "bacteria" or "fungi" or "yeast" or "*Candida*") AND ("bites" or bitemark or "bite mark"). This query intended to respond to the following PICO question: In human victims presenting human bite marks, how can microbiology assist forensic science through the analysis of the transmission of microorganisms between the bite mark and the oral cavity of the aggressor as a tool for the identification of the biter.

First, articles that corresponded to reviews, systematic reviews, and meta-analyses were excluded. The selection of articles was made progressively, starting by reading the title, then the abstract, and, finally, by reading the full article. The eligibility assessment of each article was made independently by the three authors and disagreements were resolved by consensus, excluding all those who did not meet the established inclusion criteria.

Data were extracted from each primary study by the review authors and organised into a table, including title, authors, and year of publication, with the variants defining the population (number and type of participants), the type of study, the main objective, the intervention (microbial group assessed and method of analysis), and the outcome (major findings and quantitative results).

For risk of bias analysis in the individual studies, the Joanna Briggs Institute-Faculty of Health and Medical Sciences at the University of Adelaide protocol was followed.[13] This analysis was conducted by the authors separately, and articles were classified as to whether the risk of bias is "no", "yes" or "unclear" for each question present in the protocol, in all included articles. For each yes, a point was given, and articles scoring 6 or over were selected for this review.

RESULTS AND DISCUSSION

A total of 42 articles was obtained with the database search but only 6 were included for analysis and data extraction (Fig. 1). All articles presented a low risk of bias analysis and, therefore, were included in the review (Supplementary Table 1). The 6 articles selected were experimental studies published in English, 4 papers used volunteers performing self-inflicted bite marks[1,2,5,6] and 2 papers used only saliva samples.[3,4] The ability to recover and amplify microbial DNA and, subsequently, its reliability to distinguish between individuals and match each sample to the perpetuator was evaluated in the 6 studies, in which the genus *Streptococcus* was the selected microbial group. However, the methods of analysis vary between articles and each study presented extra specific objectives (Table 1).

In the study by Kennedy et al.[1] bite marks and teeth (upper and lower anterior teeth) were swabbed from 16 volunteers who self-inflicted bites on their upper arms. DNA was directly extracted, purified, amplified, and pyro-sequenced for 16S rRNA gene; 16S–23S rRNA intergenic spacer region (ITS); endoribonuclease P (rnpB); and RNA polymerase betasubunit (rpoβ) loci. The results demonstrated that the

analysis of the rpoβ is more likely to correctly distinguish samples than the pyro-sequencing of streptococcal 16S ribosomal RNA (16S rRNA) or the 16S-23S intergenic spacer (ITS). The 3 streptococcal DNA regions analysis to distinguish the participants showed that the probability of matching correctly between the bite marks and the teeth was 92% for ITS, 99% for 16S rRNA, and 100% for rpoβ, with a confidence interval of 95%. The species identified in bite mark and teeth samples and in all the 3 loci were *Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus cristatus*. The species *S. mitis* was the most prevalent on the teeth surface, being responsible for the difference in values obtained, since the ropβ primers are specific to this bacterium while the 16S rRNA and ITS primers are comprehensive to other species. That said, the robustness of ropβ is due to the ability to distinguish participants by the exclusive analysis of a species with profound genotypic diversity, presenting a specificity of 100%.

Figure 1. Flow diagram leading to selection of the articles

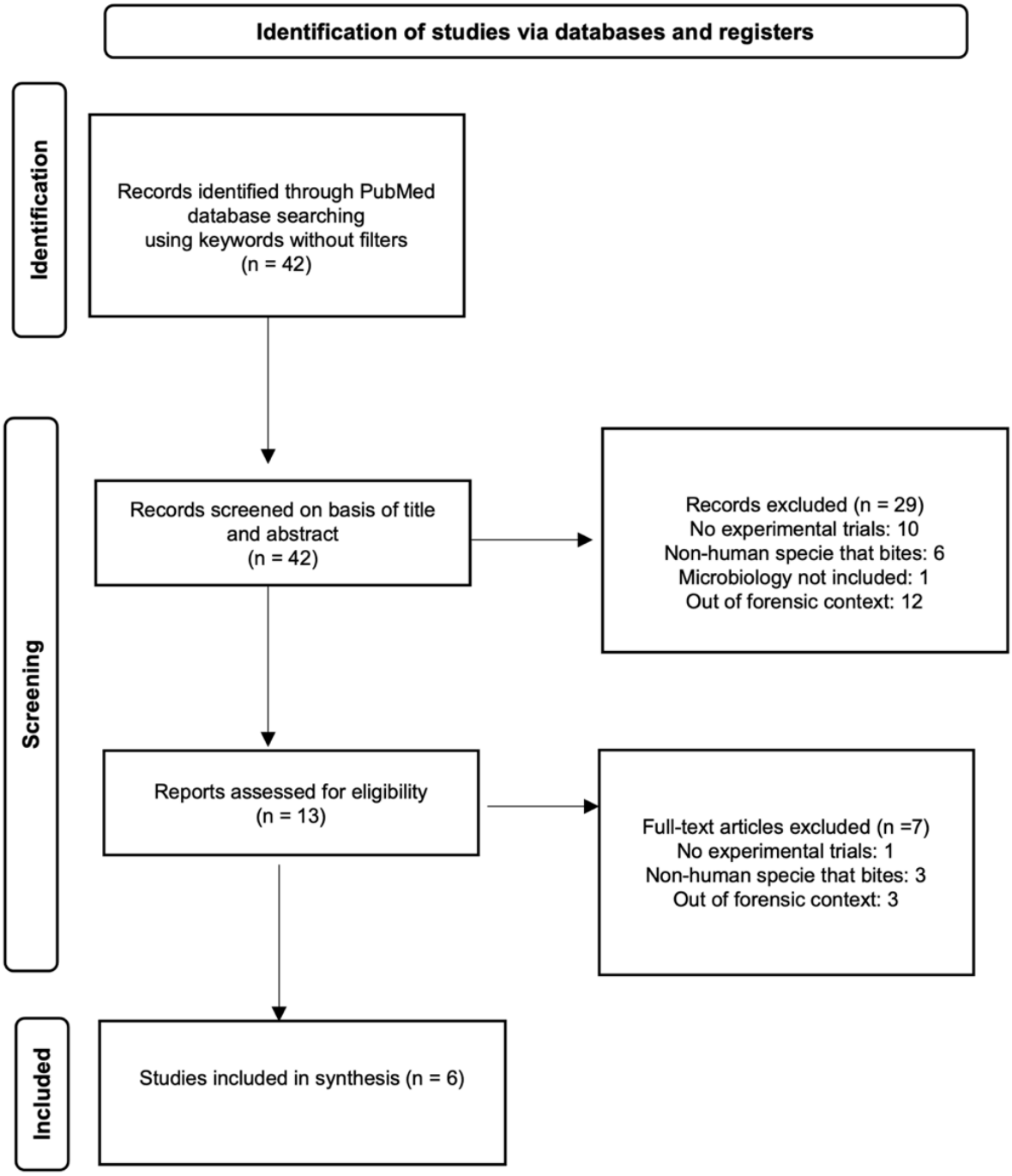

Table 1. Summarisation of the information obtained from the articles under analysis

Article	Participants	Species that bites	Main goal of study	Microbial group evaluated	Method of microbial assessment	Major findings	Identification
Kennedy et al.[1]	16 adults	Human	Capability of 3 genomic regions of streptococcal DNA to discriminate between participant samples.	*Streptococcus*	Pyro-sequencing of streptococcal 16S ribosomal RNA (16S rRNA) gene, 16S–23S intergenic spacer (ITS) and RNA polymerase beta subunit (rpoB)	Streptococcal DNA is capable of matching a bite mark to the teeth responsible. The probabilities of correctly distinguishing matching and non-matching teeth samples were 0.92 for ITS, 0.99 for 16S rRNA and 1.0 for rpoB. None of the skin control samples obtained prior to biting generated detectable amplicons using the streptococcus-specific fusion primers. Identified oral streptococci (*S. mitis, S. oralis and S. cristatus*).	Yes
Rahimi et al.[6]	8 volunteers	Human	Matching oral streptococci recovered from human bite marks with those from teeth.	*Streptococcus*	AP-PCR	400 colonies were analysed to yield a total of 106 genotypically distinguishable streptococcal strains. Between 8 and 23 genotypes recovered from each participant. The 2 most dominant genotypes from each individual composed more than 35% of all isolates from that site. Between 20% and 78% of bacterial isolates recovered at the start of the study were genotypically matched with isolates recovered 12 months.	Yes
Hsu et al.[5]	24 adults	Human	Explore the feasibility of directly amplifying bacterial DNA from bite marks from comparison with that from teeth.	*Streptococcus*	16S rDNA PCR	Streptococcal DNA can be amplified directly from bite marks. 8 of 15 bite mark amplicon patterns were matched to the corresponding incisor samples by correlation coefficients greater than 0,70 with one pairing scoring 1.0. The highest correlation between incisor amplicon profiles as 0,57, giving an indication of the level of co-incidental similarity between unrelated profiles.	Yes
Borgula et al.[2]	8 volunteers	Human	Matching *Streptococcus* isolates recovered from bite marks with the incisor teeth.	*Streptococcus*	DNA fingerprinting	Bacteria can be recovered from bite mark impress on human skin and could be matched exclusively to the teeth perpetrator. Oral streptococci recoverable decreased 30.8%/h over the first 3 hours and 5-6%/h between 6 and 24h. Manual rubbing decreased -80%; moderate physical exertion for 10 minutes decreased more than 95%. Streptococci were not recovered from control fabric squares that had not been bitten. 58 genotypes isolated from the teeth and 54 from bite marks. 60 distinct patterns were identified. Between 2 and 8 genotypically distinguishable strains were isolated from each tooth and bite mark.	Yes
Elliot et al.[4]	Saliva (2 donors)	Human	Distinguishing between oral isolates of S. *salivarius* from 2 persons.	*Streptococcus salivarius*	Pyrolysis mass spectrometry (PY-MS)	Differentiation of *S. salivarius* at strain level according to the origin of the isolate when we have different individuals. 78 spectra were generated. Major cluster which includes the three reference strains of *S. salivarius*	Yes
Brown et al.[3]	Saliva (1 donor)	Human	Determining if is possible to use "fingerprint" identification of oral bacteria.	*Streptococcus*	M-S agar plate	Suitable "fingerprint" typing scheme for oral bacteria may provide evidence relating to the identity of a suspect in such cases. Total counts decreasing at a rate of 44.8%/h and for *S. salivarius* 43.9%/h. Recoverable streptococci after 6 hours are still large	Yes

The study by Rahimi et al.[6] intended to evaluate the efficiency of AP-PCR (Arbitrarily primed polymerase chain reaction) to identify the biter, to assess the natural distribution of oral *Streptococcus* genotypes, and to examine their recoverability after 12 months. In this study, one of the 8 volunteers, whose identity was withheld from the laboratory investigator, and one extraneous individual, firmly bit their own upper arms with sufficient force to produce indentations that lasted for at least 10 minutes. The bite marks were covered with loose clothing for 6 hours and the area impacted by the mandibular incisors was swabbed with a moistened cotton-tipped applicator for further DNA analysis. Bacterial DNA samples from the 8 volunteers were obtained by swabbing the lower incisors incisal surface. All samples were cultured, and the Streptococcal genotypes were obtained from 50 randomly selected bacterial colonies from each sample. The analysis of the streptococcal DNA allowed to distinguish 106 genotypes, and, in each individual, 8 to 23 distinct strains were found. The bacteria were unambiguously matched to the biter by comparing the amplicon profiles with those from the 8 participants. In contrast, bacteria from an additional bite mark (extraneous individual) could not be matched to any of the 8 participants. The temporal stability of the *Streptococcus* genotypes was also evaluated, and an additional sample of the incisors from each participant was collected for analysis after 12 months; results showed that 20% to 78% of the catalogued bacterial genotypes were recovered after this period. Moreover, throughout the study period, none of the bacterial genotypes was shared between participants. This study demonstrated that the AP-PCR method facilitates a faster analysis of a large number of bacteria with no evident loss of resolution presenting discriminating power to be used in a forensic context. This approach to bite mark analysis may have an immediate application for individual identification, linking a suspect to the crime, within a limited number of individuals.

The study by Hsu et al.[5] intended to explore the consistency of the direct amplification of bacterial DNA recovered from the bite mark for comparison with oral samples. The streptococcal DNA was obtained from self-inflicted lesions by each of the 24 participants (after 3 hours and from the lower incisors), from unbitten control sites, adjacent to the bite marks, and from the lingual surfaces and lower incisors incisal surfaces of the biters. The Streptococcal DNA was amplified by PCR using primers specific for streptococcal 16S rDNA. The comparison of amplicon profiles was done by denaturing gradient gel electrophoresis (DGGE). Amplicon patterns generated from bite marks with 6 or more bands of DNA were compared with the incisors, where 8 (out of 15) coincided with the corresponding incisor, with a correlation coefficient greater than 0.70. This study provides support for a microbiologically based approach to the analysis of bite marks, using streptococcal DNA amplified directly from the bite mark.

The work of Borgula et al.[2] aimed to evaluate the feasibility of recovering oral *Streptococcus* from bite marks on human skin and clothing and the reliability in the correspondence to the *Streptococcus* collected from the incisors responsible for the bite, using genomic comparison. The samples were collected from self-inflicted lesions in the arms of the 8 volunteers, from various fabrics, and from the biter's lower incisors, cultured and the genomic profiles of the recovered bacteria analysed by DNA "fingerprints". After their analysis, it was concluded that recoverable oral streptococci of the skin decreased exponentially over time, namely by 30.8% per hour in the first 3 hours and 5% - 6% per hour between 6 and 24 hours after the bite. Of the 8 volunteers, 58 *Streptococcus* genotypes isolated from the teeth and 54 *Streptococcus* genotypes from the bite mark were compared, and, from each individual, it was possible to distinguish between 2 to 8 chains of each bite mark and dental source. It also demonstrates oral bacteria can be recoverable from the bite marks imprinted on the skin for up to 24 hours. Due to the extreme genotypic diversity of oral streptococci, the microorganisms recovered from the bite marks can correspond exclusively to the responsible tooth in each of the 8 samples, indicating that this approach can support the identification of the suspect of a crime, involving a bite mark.

In the study by Brown et al.,[3] the possibility of recovering up to 6.25h *Streptococcus salivarius* from ten-microlitre aliquots of whole saliva applied to human skin was evaluated. The saliva was plated in a selective medium, Mitis-Salivarius agar, to identify these bacteria and, subsequently, establish the time that *S. salivarius* can be recovered on human skin. The results of these

experiments showed a decrease of 44.8% per hour in the total number of *Streptococcus* and 43.9% per hour in *S. salivarius*; however, after 6 hours there is still a large amount of recoverable microbial material. Thus, the establishment of a suitable fingerprint typing scheme for oral bacteria may provide evidence relating to the identity of a suspect in such cases.

The study by Elliot et al.[4] had as its main objective to evaluate the applicability of Pyrolysis mass spectrometry (Py-MS) to distinguish samples of isolated *Streptococcus salivarius*, obtained from saliva samples of 2 different individuals. After collecting the saliva samples, the bacteria were cultured and isolated and submitted to Py-MS, where 78 spectra were obtained. This allowed authors to conclude that the analysis of the samples of *S. salivarius* by this method was able to distinguish between 2 different individuals.

Overall, all selected studies have shown that the analysis of the oral microbiome, particularly the genus *Streptococcus*, has the potential to be used in a forensic investigation, since, in theory, it is possible to match the bacterial profile from the bite mark found on human skin with that obtained from the teeth, and, therefore, connect an injury to the aggressor. In addition, it was possible to estimate the decrease in the recovery of the oral *Streptococcus* in a given period of time, and the rate of decay can exceed 30% in the first 6 hours,[2,3] demonstrating the importance of a rapid collection and storage. Moreover, it was also shown that, if the samples were collected and packaged correctly, the genotypes can be analysed after 12 months, demonstrating temporal stability of the oral streptococci.[6] Notwithstanding, further research should be done with a significantly higher number of participants.

The genus *Streptococcus* belongs to the most predominant classes in the oral microbiota, with a presence of about 20% in the salivary microbiota and 15% in plaque, and presenting a high genetic variability.[14] These reasons justify the focus of the studies analysed in this review on *Streptococcus* genus with interesting positive results. However, this may constitute also a limitation since the oral microbiota includes several other genus and species that could be explored as forensic tools as well.

Regarding the methods, 2 older papers only cultured samples in Mitis-Salivarius agar,[3,4] 2 papers compared the genotype profiles of isolated strains obtained after culturing samples in Mitis-Salivarius agar,[2,6] and the other 2 papers extracted streptococcal DNA directly from samples without culturing.[1,5] Previous studies have stated that culture-independent molecular methods show more promising results, better demonstrating the composition and variety of the oral microbiota,[15] since bacterial diversity in most environments is severely underestimated in surveys with culture-based techniques.[16] In many natural environments, less than 1% of organisms are culturable.[17] Due to the significant effort put into culturing oral bacteria, it is thought that about 50% of oral bacteria have been cultivated.[18] Therefore, in the studies where culture-dependent methods of analysis were used,[2-4,6] the results may not fully identify and characterise all the microbiota present in the samples, representing a limitation of these studies.

The different studies used different methods for the genotyping analysis of *Streptococcus*, namely amplification of specific genes (including 16S rRNA) and direct pyro-sequencing,[1] DGGE of 16S rRNA,[5] AP-PCR,[6] or whole genomic "fingerprinting" using several restriction endonucleases.[2] In a forensic context, most of samples have relatively low bacterial levels and require highly sensitive methods,[19] so it is, therefore, necessary to adopt standardised lab protocols (collection and analysis of samples)[20,21] and bioinformatics work,[22] enabling the removal of the biases associated with different extraction protocols, PCR reactions, and sequencing platforms[19] and to integrate the information and allow easy communication across studies.[23] Interesting to note, the study by Kennedy et al.,[1] showed that, in comparison with 16S rRNA and ITS, ropβ analysis appear to be the most robust method with the highest specificity. So, future studies should further explore these genetic markers as a possible relevant tool in bite marks analysis.

The studies focusing only on the saliva analysis[3,4] also demonstrated that saliva *Streptococcus* presents a relevant inter-individual variability regarding the streptococci profile, important for the forensic application of this microbial oral group. However, these older studies presented a significant lack of sensibility in species discrimination. Notwithstanding, the more recent studies using more discriminatory genetic tools also demonstrated a high discriminatory rate in what concerns perpetrator identification,

despite the reduced scope of participants evaluated in each study. Therefore, future studies with a larger number of individuals are necessary to validate oral *Streptococcus* as a relevant forensic tool.

Despite the positive results obtained, the studies also share several limitations that may not reflect the real conditions in which this analysis may be used, since there are factors, such as body lotions, antibacterial mouth washes, or body secretions, that can affect human microbiota, both on skin and oral cavity.[2] Also, in crime situations, some common behaviours associated with the victims, such as rubbing or washing the skin,[24] may negatively influence the survival of the bacterial community in the injuries that are inflicted on them during biting.[2]

There were some limitations of this study, namely in what performing a metanalyses is concerned. In fact, as the 6 selected studies had different goals and different methodologies, this kind of statistical approach was not an option.

CONCLUSIONS

This systematic review highlights that the microbiological evidence taken from bite marks may provide important information in the forensic investigation due to its ability to match the bacteria in the oral cavity, in particular from *Streptococcus* genus, with the bacteria recovered from the biter's mouth. However, much more research is still needed before this forensic strategy can be applied in real scenarios. There is still a need to optimize and standardize the methods of analysis and to determine several factors that may influence the results, such as the frequency of bacterial genotypes in the human population and the temporal stability of the oral microbiome in human skin.

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Supplementary Table 1. Risk of bias analysis

	1. Were the criteria for inclusion in the sample clearly defined?	2. Were the study subjects and the setting described in detail?	3. Was the exposure measured in a valid and reliable way?	4. Were objective, standard criteria used for measurement of the condition?	5. Were confounding factors identified?	6. Were strategies to deal with confounding factors stated?	7. Were the outcomes measured in a valid and reliable way?	8. Was appropriate statistical analysis used?	Overall appraisal
(Kennedy et al., 2012)	Yes	Yes	Yes	Yes	No	Not applicable	Yes	Yes	Include
(Rahimi et al., 2005)	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Include
(Hsu et al., 2012)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Include
(Borgula et al., 2003)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Include
(Brown et al., 1984)	Yes	Yes	Yes	Yes	No	Not applicable	Yes	Yes	Include
(Elliot et al., 1984)	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Include

Note: Articles were classified as to whether the risk of bias is "no", "yes" or "unclear" for each question present in the protocol, in all included articles. For each yes, a point was given, and articles scoring six or over were included in this review.