SYSTEMATIC REVIEW AND META-ANALYSIS OF DATA ON NITRATE REDUCTASE ASSAY (NRA) FOR RAPID DIAGNOSIS OF DRUG-RESISTANT TUBERCULOSIS

Report prepared by Dr Anandi Martin, 1 July 2009

Institute of Tropical Medicine, Mycobacteriology Unit, Antwerp, Belgium

Summary

Early detection of drug resistance in tuberculosis (TB) allows the use of appropriate treatment regimens for the patient, which has an important impact for the better control of the disease. The development of rapid methods for drug susceptibility testing (DST) is very important due to the increasing rates of multidrug-resistant tuberculosis (MDR-TB) worldwide and the recently described extensively drug-resistant tuberculosis (XDR-TB). The reference standard methods for DST of M. tuberculosis are very slow to give results, and due to the emergence of multidrug-resistant tuberculosis there is an urgent demand for new, rapid and accurate DST methods, particularly in low-income countries. The commercial liquid-medium MGIT 960 reduces the turnaround time but is expensive and places higher demands on equipment to be routinely used in poor-resource countries. Among the phenotypic methods proposed, the nitrate reductase assay (NRA) is a simple technique based on the capacity of M. tuberculosis to reduce nitrate to nitrite, which is detected by adding a reagent to the medium. NRA is a non-commercial method and has received increasing attention because of its simplicity and the absence of any requirement for sophisticated equipment or highly trained personnel. Several studies have evaluated its accuracy and performance in comparison with reference standard methods, particularly for the detection of resistance to rifampicin and isoniazid, which are the two most important drugs used for the treatment of TB.

There is evidence that NRA is highly sensitive and specific for the rapid detection of rifampicin and isoniazid resistance in culture isolates and seems to be good method to use in laboratories that lack access to other more sophisticated techniques, as is often the case in laboratories in limited-resource countries.

Systematic review of NRA has been done in 2007 to synthesize all the published evidence of this method. WHO has recently developed a systematic, structured process evidence-based policy development on the use of new diagnostic tools, with the aim to facilitate rapid policy guidance on introduction of contemporary tools into national TB control programmes. The objective of this study is to make an update of the NRA systematic review of available data, using standard methods appropriate for diagnostic accuracy studies according to the WHO policy.
**Introduction**

Early detection of drug resistance in tuberculosis (TB) allows the use of appropriate treatment regimens for the patient, which has an important impact for the better control of the disease. The development of rapid methods for drug susceptibility testing (DST) is very important due to the increasing rates of multidrug resistant tuberculosis (MDR-TB) worldwide and the recently described extensively drug resistant tuberculosis (XDR-TB) (Shah et al., 2007; Aziz et al., 2006). The World Health Organization (WHO) and members of the STOP TB Partnership called for urgently expand access to culture and DST in response to the spreading out of MDR-TB and XDR-TB and declared by the WHO as a serious emerging threat to public health (WHO 2007). This poses significant challenges for TB laboratory capacity and the need for faster DST methods. Conventional culture methods using egg- or agar-based media are still the most commonly used approach in many countries. To test for drug resistance, the standard methods using Löwenstein-Jensen (LJ) medium include the proportion method, the absolute concentration method and the resistant ratio method, which are well standardised with clinical isolates, at least for the major antituberculosis drugs. The proportion method was developed in the 1960s and is still “the gold standard method” used in many laboratories especially in developing countries because it is an inexpensive method easily accessible in these settings (Canetti et al., 1963; 1969). During the last years, due to the long turnaround time of conventional DST methods, several new approaches have been proposed for faster detection of MDR-TB including both genotypic and phenotypic methods (Palomino et al., 2005; 2006). Among the phenotypic methods proposed, the nitrate reductase assay (NRA) is a simple technique based on the capacity of *M. tuberculosis* to reduce nitrate to nitrite, which is detected by adding the Griess reagent to the medium. By incorporating 1 mg/mL of potassium nitrate (KNO$_3$) in the LJ medium, the reduction of nitrate can be detected using the Griess reagent, which produces a coloured reaction. When in the presence of rifampicin or isoniazid at the critical concentration, the appearance of a red-pink colour represents resistance to the drug. Susceptible strains will lose the capacity to reduce nitrate producing no coloured reaction as they are inhibited by the antibiotic. Results can be obtained faster than by visual detection of colonies since the NRA uses the detection of nitrate reduction as an indicator of growth. Progress has been made in the use of the NRA for DST in *M. tuberculosis* in the last five years. However, it is essential to further evaluate this new procedure.
before bringing it into routine laboratory diagnosis. We conducted a systematic review and meta-analysis to synthesize all available literature on the NRA for DST in *M. tuberculosis* to evaluate the overall accuracy of this method for the detection of MDR-TB in isolates and in sputum samples.

**Method**

**Literature search**

The most commonly employed search strategy used Medline PubMed (NCBI) database. Additional databases were also included such as Global health-CAB, EJS-E (EbscoHost), ISI Web, Web of Science and IFCC. Search terms (free text, keywords) were “nitrate reductase”, “griess”, “nitrate test”, “nitratase”, “Mycobacterium tuberculosis”, “tuberculosis” “drug susceptibility”, “drug resistance”, “multidrug resistance” “diagnosis”, “rifampicin”, “rifampin” “isoniazid” for papers published in English from 1966 onwards. All retrieved titles and abstracts were scrutinised for relevant studies about drug resistance detection of *M. tuberculosis* using the NRA.

**Study selection**

The search through electronic databases returned studies using the NRA for rapid DST in *M. tuberculosis*. We identified results from all primary studies evaluating the accuracy (sensitivity and specificity) of the NRA for rapid detection of rifampicin and isoniazid resistant tuberculosis in *M. tuberculosis* isolates or in sputum samples. We included studies that met the following pre-determined criteria: comparison of the NRA with a reference standard method (including proportion method, absolute concentration method, resistance ratio method or radiometric BACTEC 460-TB method), detection of rifampicin and isoniazid resistance; studies that reported data on false-positive, true-positive, false-negative, and true-negative results. Our initial search had no language restrictions but studies not available in English language were excluded from the data extraction process. The heterogeneity of data was addressed by performing a subgroup analysis with the NRA performed on isolates, in solid or in liquid medium, and on sputum samples.
Data extraction

Two independent reviewers examined the titles and abstracts of all identified studies to confirm they had fulfilled the above defined inclusion criteria. Titles and abstracts were first read independently by the two reviewers and then all papers considered possibly eligible were reviewed independently by the authors who assessed whether the paper was concerned with the NRA for DST. The bibliographies of selected articles were screened for potentially suitable references which were then retrieved. Those studies that did not match with our requirements were taken out. Data of each article were extracted by one reviewer and a sample of these was assessed by a second reviewer to check accuracy of data extraction. Articles were examined in detail and any disagreement was resolved by consensus with a third author. We classified data according to the following parameters included in tables 1 and 2: the reference standard method used, type of sample (isolates or sputum), the sample size, the outcome data (sensitivity and specificity as determined by comparison with the reference standard), the time to positivity (TTP) that evaluates the speed of the NRA, which means on how many days results were available.

For each included study, data were also extracted to generate a two by two table for estimating the sensitivity and the specificity of the NRA. All extracted data were double checked by a second author. Sensitivity (true positive rate, TPR) was defined as the proportion of isolates determined to be rifampicin or isoniazid resistant by the reference method correctly identified as rifampicin or isoniazid resistant by the NRA method. Specificity (true negative rate or false positive rate, FPR) was defined as the proportion of isolates determined to be rifampicin or isoniazid susceptible by the reference method correctly identified as rifampicin or isoniazid susceptible by the NRA method.

Data synthesis and meta-analysis

We performed the meta-analysis in accordance with published guidelines (Walter 2005; 2002) and performed data analysis using the Meta-DiSc software (version 1.4) (Zamora et al., 2006).

We created a forest-plot to estimate the accuracy of each test and the receiver operating characteristic (ROC) curves that are well established as methods for summarizing the performance of a diagnostic test within a single study. It indicates
the relationship between the true positive rate (TPR) and the false positive rate (FPR) of the test. The summary receiver operating characteristic (SROC) curve is similar to the ROC curve for a single study except that the data points for the SROC curve are obtained from a set of studies being used for an overview and meta-analysis. The area under the curve (AUC) represents an overall summary of the performance of a test. AUC ranges from 1 for a perfect test that always correctly diagnoses, to 0 for a test that never correctly diagnoses. The Q* index represents a summarization of test performance where sensitivity and specificity are equal. The heterogeneity among studies were analysed using the heterogeneity chi-squared and $I^2$ index (interpreted as the percentage of the total variability in a set of effect sizes due to true heterogeneity) included in Meta-DiSc program (Huedo-Medina et al., 2006).

**Quality assessment**

We assessed study quality of individual studies using the criteria based on the QUADAS tools for assessment of quality of diagnostic studies (Whiting et al., 2003). See Tables 3 and 4 (Supplementary data).

**Results**

**Description of studies**

**Detection of rifampicin resistance**

The search through electronic databases returned 22 studies using the NRA for the rapid detection of rifampicin and isoniazid resistance in *M. tuberculosis*. 17 studies reporting results of rifampicin resistance detection met eligibility criteria and are included in this review. Two additional unpublished studies are included after having contact with the author (Shikama et al., 2009; Senia et al., 2009). We did not consider studies that did not compare the assay with a reference standard method (Kumar et al., 2005, Sanchotene et al., 2008) or that use NRA for the implementation of the method in the field under programme condition (Asencios et al., 2008; Yagui et al., 2006; Shin et al., 2008). Table 1 describes the characteristics and outcomes of these studies. Fifteen studies performed the NRA on culture isolates and four on sputum samples. From the 15 that performed the NRA on isolates, three used a liquid medium format (Syre et al., 2003; Kumar et al., 2005; Poojary et al., 2006)
and one study that performed the test directly on sputum sample uses also a liquid medium (Affolabi et al., 2008 (a)). Studies are recorded in the table in order to describe the outcome of the subgroup analysis. All studies were published between 2002 and 2009. Figure 1 illustrates the forest plots that estimate the sensitivity and specificity based on results of all the included studies. For the majority of the studies, sensitivity has been reported between 94% and 100%, a pooled sensitivity of 97% was found for RIF (figure 1) and the specificity was exceptionally reported as 100% for the majority of the studies. Figure 2 shows the SROC curve for the same data and shows an AUC of 0.99 and Q* of 0.97, indicating a high level of overall accuracy.

Subgroup analysis of accuracy of NRA on isolates-solid and liquid medium
We performed a subgroup analysis of the studies that applied the test on isolates or on sputum samples separately. As it can be seen in figure 1, the majority of the studies that performed the NRA on isolates had sensitivity and specificity of 100%. Studies that used a liquid medium format (Syre et al., 2003; Kumar et al., 2005; Poojary et al., 2006) had a sensitivity and a specificity higher than 94% and had an average TTP of 6 days compared to 8-10 days of those performed the NRA on solid medium (Table 1).

Subgroup analysis of accuracy of NRA on sputum samples
As illustrated in figure 1, the four studies that applied the NRA directly on sputum samples (Musa et al., 2005; Solis et al., 2005, Affolabi et al., 2007, Affolabi et al., 2008) reported sensitivity between 87% and 100%. The lowest sensitivity, however, was reported in a study with only seven true resistant isolates out of which one was missed with the NRA (Affolabi et al., 2007). Specificity was still high at 100% and TTP ranged from 14 to 21 days. One study performed the test directly on sputum sample using a liquid medium and show good results with a sensitivity and specificity of 100% and 99% respectively (Affolabi et al., 2008).

Detection of isoniazid resistance
Table 2 describes the characteristics of the 17 studies that reported results for isoniazid resistance detection. Four studies performed the NRA directly on sputum samples (Musa et al., 2005; Solis et al., 2005, Affolabi et al., 2007, Affolabi et al., 2008). Figure 3 shows the forest plot that estimates the sensitivity and specificity based on results of all the studies. The sensitivity was between 87% and 100% and the
specificity was higher between 95% and 100%. Figure 4 shows the SROC curve indicating an AUC of 0.99 and a Q* of 97 indicating also a high level of overall accuracy.

**Subgroup analysis of accuracy of NRA on isolates-solid and liquid medium**

Ten studies that performed the NRA on isolates in solid medium had a sensitivity that ranged between 92% and 100% (Angeby et al., 2002; Coban et al., 2004; Sethi et al., 2004; Montoro et al., 2005; Martin et al., 2005; Mengatto et al., 2006; Lemus et al., 2006; Shikama et al., 2009 a&b). TTP of these studies was between 7 and 14 days (table 2). As for the detection of rifampicin resistance, studies that used a liquid medium format (Syre et al., 2003; Kumar et al. 2005; Poojary et al., 2006) had an average TTP of 6 days (Table 2).

**Subgroup analysis of accuracy of NRA on sputum samples**

As illustrated in figure 3, four studies (Musa et al., 2005; Solis et al., 2005, Affolabi et al., 2008 a&b) tested NRA directly on sputum samples to detect resistance to isoniazid and the sensitivity was reported between 93% and 99%. Specificity was close to 100% in the four studies. The TTP ranged between 7 and 21 days (table 2). Only one study (Afflobai et al., 2008 b) performed the NRA on sputum sample using a liquid media and found sensitivity and a specificity of 100% with a TTP between 8-14 days.

**Discussion**

The goal of DST is the early detection of drug resistance, especially to rifampicin and isoniazid, the two most effective drugs currently available for the treatment of TB. This allows early detection of MDR-TB and a better management and treatment of patients. Early identification of MDR-TB cases would decrease the risk of disease and possible amplification of drug resistance. The methods currently available for rapid DST of *M. tuberculosis* are cheap but slow or fast but too costly to be applicable in most high incidence TB areas. There is obviously a great need for fast, reliable, and inexpensive methods for DST of *M. tuberculosis*. However, any new rapid DST method must be carefully calibrated with representative isolates of *M. tuberculosis* in order to determine in vitro the cut-off of resistance and sensitive isolates with acceptable reproducibility.
The present meta-analysis suggests that the NRA is highly sensitive and specific for detecting rifampicin and isoniazid resistant TB both in culture isolates and directly on sputum samples. The majority of studies had sensitivity of 95% or greater, and nearly all were 100% specific. Even with subgroup analysis, all studies yielded consistently high estimates of sensitivity and specificity, so heterogeneity was not affected in this meta-analysis. The NRA has shown a high degree of accuracy when used on culture isolates but this requires 2–6 weeks for primary isolation of the bacteria. Only four studies have applied the NRA directly on sputum samples for RMP and INH resistance detection. Additional studies are needed to better establish the accuracy of NRA applied to sputum, but preliminary studies suggest that NRA maybe useful for rifampicin and isoniazid resistance detection in sputum samples. Accuracy was in general slightly higher for rifampicin resistance detection, however, isoniazid resistance detection results also showed high sensitivity and specificity even when applied in sputum samples.

All studies have reported results showing that the NRA is a faster method compared to the conventional method. The average time to positivity was between 7-14 days when performed on isolates and between 7 and 21 days when used directly on sputum samples, compared to the reference standard method that takes 4 to 6 weeks when performed on LJ or 3 weeks when performed on agar with 2-3 additional weeks for the first isolation.

The great advantage of the NRA is that it is performed on the classical LJ medium that TB laboratories use routinely for diagnosis of TB. This is a very important factor because laboratories do not have to change completely to another new method since adaptation to a novel technology is not always easy to implement in laboratories involved in routine work. No equipment is required to perform the NRA giving the opportunity for its widespread application. Results are simple to interpret by a change of colour. Also biosafety problems are limited since the test is performed on a solid medium reducing the risk of production of aerosols during manipulation. A limitation of the NRA is that this method cannot be used for nitrate reductase negative *M. tuberculosis* samples but on the other hand nitrate reductase negative strains of *M. tuberculosis* are unusual. DST would not be possible to be performed for *M. bovis*.

We are confident that our analysis did not miss any major study recorded in the databases searched. One weakness of this review might be that we excluded studies
not available in other languages than English. In 2002, Panaiotov et al., in Bulgaria, described the NRA for DST of *M. tuberculosis* strains. Results obtained were in accordance with the results of the proportion method and were obtained within 8-10 days. The technique was in use since 1980 by Emil Kalfin from the National Center of Lung Disease in Bulgaria. Between 1998 and 2000 the same team modified and improved the NRA with the use of a crystalline nitrate reductase reagent. This crystalline reagent is reported to be less toxic and to have a longer shelf-life than the Griess reagent. In 2003, Golyshevskaia et al., in an article published in Russian, compared the NRA with the BACTEC 960 system for the detection of resistance to first-line drugs. In 2006, Poliakov et al., in Russia performed NRA directly on sputum samples for the detection of rifampicin and isoniazid resistance. After reviewing the abstracts of these papers it suggests that the overall results are similar to the results of the studies included in this analysis.

The overall quality of the included studies was good according to the analysis performed with the QUADAS tool.

**Conclusion**

NRA has been shown to be highly sensitive and specific in the detection of rifampicin and isoniazid resistance when used on clinical isolates. With the objective to reduce the TTP, NRA performed directly on sputum smear-positive samples saves valuable time by omitting the pre-isolation step. Additional research is needed to better establish the accuracy of the NRA applied directly on sputum samples since very few studies are reported in the literature. However, available data of these studies show that the NRA is promising to be applied directly on sputum samples. Studies are also needed to know the performance of the test in countries with a high prevalence of MDR-TB or in a population in which MDR-TB is suspected. Finally, additional studies are needed to establish the cost-effectiveness of the NRA as compared to the conventional methods demonstrating the benefit of this technology.

For rifampicin, the majority of the studies had sensitivity and specificity greater than 94% and for isoniazid, greater than 92%. The four studies that applied NRA directly on sputum samples had a sensitivity and specificity that ranged between 87% and 100%. The SROC curve had an area of 0.99 for both drugs. There is evidence that NRA is highly sensitive and specific for the rapid detection of rifampicin and isoniazid resistance in culture isolates. More evidence is needed for NRA applied
directly on sputum samples but preliminary results appears promising and shows to have a good sensitivity and specificity. Additional cost-effectiveness analysis is still needed.
References


Song F, Khan KS, Dinnes J, et al. Asymmetric funnel plots and publication bias in meta-analyses of diagnostic accuracy. *Int J Epidemiol* 2002; **31**:88-95


Walter SD. The partial area under the summary ROC curve. *Statistic in medicine* 2005; **24**: 2025-2040.


Table 1: Description of studies included in meta-analysis for rifampicin resistance detection

<table>
<thead>
<tr>
<th>Authors, publication year</th>
<th>Country</th>
<th>Number clinical isolates</th>
<th>Reference test</th>
<th>Sample size (# resistant/# sensitive)</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
<th>TTP</th>
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</thead>
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<tr>
<td>Angeby et al., 2002</td>
<td>Sweden</td>
<td>57</td>
<td>BACTEC 460</td>
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<td>1.00 (0.88-1.00)</td>
<td>1.00 (0.87-1.00)</td>
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<td>Syre et al., 2003</td>
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<td>73, liquid</td>
<td>BACTEC 460</td>
<td>16/57</td>
<td>0.94 (0.71-0.99)</td>
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<tr>
<td>Lemus et al., 2004</td>
<td>Cuba</td>
<td>20</td>
<td>PM LJ</td>
<td>10/10</td>
<td>1.00 (0.69-1.00)</td>
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<td>1.00 (0.95-1.00)</td>
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<td>190</td>
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Table 2: Description of studies included in meta-analysis for isoniazid resistance detection

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**Figure 1.** Forest plot of the sensitivity and specificity for rifampicin.

a) Sensitivity RIF

The point estimates of sensitivity and specificity from each study are shown as circles. Error bars are 95% confidence intervals.
b) Specificity RIF

Specificity (95% CI)

- Angeby 2002: 1.00 (0.87 - 1.00)
- Syre 2003: 1.00 (0.94 - 1.00)
- Lemus 2004: 1.00 (0.94 - 1.00)
- Coban 2004: 1.00 (0.95 - 1.00)
- Sethi 2004: 1.00 (0.94 - 1.00)
- Montoro 2005: 1.00 (0.94 - 1.00)
- Martin 2005: 1.00 (0.95 - 1.00)
- Musa 2005 (sputum): 1.00 (0.97 - 1.00)
- Solis 2005 (sputum): 1.00 (0.96 - 1.00)
- Kumar 2005: 1.00 (0.89 - 1.00)
- Poojary 2006: 1.00 (0.95 - 1.00)
- Mengatto 2006: 1.00 (0.91 - 1.00)
- Lemus 2006: 1.00 (0.99 - 1.00)
- Affolabi 2007 (sputum): 1.00 (0.98 - 1.00)
- Affolabi 2008 (sputum): 0.99 (0.96 - 1.00)
- Affolabi 2008: 1.00 (0.97 - 1.00)
- Shikama 2009 (a): 0.98 (0.86 - 1.00)
- Shikama 2009 (b): 0.97 (0.84 - 1.00)
- Senia 2009: 1.00 (0.97 - 1.00)

Pooled Specificity = 1.00 (0.99 to 1.00)
Chi-square = 13.40; df = 18 (p = 0.7671)
Inconsistency (I-square) = 0.0 %
Figure 2. Summary receiver operator curve (SROC) plot for rifampicin. Each circle represents each study in the analysis. The curve is the regression line that summarizes the overall diagnostic accuracy. SROC, summary receiver operating characteristic; AUC, area under the concentration–time curve; SE (AUC), standard error AUC; Q*, an index defined by the point on the SROC curve where the sensitivity and specificity are equal, which is the point closest to the top-left corner of the ROC space; SE(Q*), standard error of Q* index.
Figure 3: Forest plot of the sensitivity and specificity for isoniazid

a) Sensitivity INH

Pooled Sensitivity = 0.97 (0.95 to 0.98)
Chi-square = 24.06; df = 16 (p = 0.0881)
Inconsistency (I-square) = 33.5 %

Sensitivity (95% CI)

- Angeby 2002: 0.97 (0.84 - 1.00)
- Syre 2003: 1.00 (0.89 - 1.00)
- Coban 2004: 1.00 (0.75 - 1.00)
- Sethi 2004: 0.97 (0.84 - 1.00)
- Montoro 2005: 0.96 (0.85 - 0.99)
- Martin 2005: 0.97 (0.88 - 1.00)
- Musa 2005 (sputum): 0.93 (0.66 - 1.00)
- Solis 2005 (sputum): 0.99 (0.95 - 1.00)
- Kumar 2005: 0.87 (0.60 - 0.98)
- Poojary 2006: 0.94 (0.79 - 0.99)
- Mengatto 2006: 0.92 (0.75 - 0.99)
- Lemus 2006: 0.92 (0.82 - 0.97)
- Affolabi 2008 (sputum): 1.00 (0.77 - 1.00)
- Affolabi 2008: 1.00 (0.85 - 1.00)
- Shikama 2009 (a): 0.98 (0.92 - 1.00)
- Shikama 2009 (b): 0.94 (0.84 - 0.99)
- Senia 2009: 1.00 (0.96 - 1.00)
b) Specificity INH

Figure 4. Summary receiver operator curve (SROC) plot for isoniazid. Each circle represents each study in the analysis. The curve is the regression line that summarizes the overall
diagnostic accuracy. SROC, summary receiver operating characteristic; AUC, area under the concentration–time curve; SE (AUC), standard error AUC; Q*, an index defined by the point on the SROC curve where the sensitivity and specificity are equal, which is the point closest to the top-left corner of the ROC space; SE(Q*), standard error of Q* index.
Table 3: The score for the QUADAS tools item for the detection of rifampicin resistance

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1 = Yes
0 = No or not applicable
0.5 = Unclear

Table 4: The score for the QUADAS tools item for the detection of isoniazid resistance
1 = Yes
0 = No or not applicable
0.5 = Unclear