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Performance of Meridian ImmunoCard *Mycoplasma* Test in a Multicenter Clinical Trial

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Serology is the principal laboratory method used to diagnose *Mycoplasma pneumoniae* infection. Meridian Diagnostics has developed the ImmunoCard *Mycoplasma* kit, a 10-min card-based enzyme-linked immunosorbent assay (ELISA) designed to detect immunoglobulin M (IgM) antibodies to *M. pneumoniae*. We compared the ImmunoCard with two *M. pneumoniae* IgM-specific assays (immunofluorescence assay [IFA] and ELISA) and a standard complement fixation (CF) procedure using 896 specimens submitted to clinical laboratories for *M. pneumoniae* serology. Equivocal results obtained by CF, IFA, or ELISA were resolved by testing with an additional method or by reviewing patient chart information. The ImmunoCard had sensitivities ranging from 74% compared with the ELISA to 96% compared with CF results resolved with IFA. ImmunoCard specificities ranged from 85% compared with the IgM-specific ELISA to 98% compared with IgM-specific IFA results resolved with clinical chart review. We also compared the ImmunoCard results with consensus results of 694 specimens tested on at least two non-ImmunoCard methods because of the lack of a "gold standard" for *M. pneumoniae* serology. Overall, the ImmunoCard *Mycoplasma* IgM assay had 90% sensitivity, 93% specificity, and 92% agreement with the consensus results. The ImmunoCard is technically less complex and requires less equipment than the three other assays. Our results indicate that the ImmunoCard *Mycoplasma* IgM assay is a valid and simple procedure which can reduce technologist time (and, thus, labor cost) and turnaround time for laboratories analyzing small numbers of specimens (<10 per batch) submitted for IgM anti-*M. pneumoniae* testing.

Mycoplasma pneumoniae is associated with up to 20% of X-ray-proven pneumonia cases (2). Although treatment of this infection is straightforward (erythromycin or tetracycline), proving *M. pneumoniae* infection in specific patients is a complex task. *M. pneumoniae* is difficult to culture and is not normally detectable in bronchial secretions (4); thus, serology is the principal method of laboratory diagnosis. *M. pneumoniae* has both lipid (7) and protein (5) antigens which elicit antibody responses in clinical infections. During the 1960s and 1970s, clinical laboratories used complement-fixing (CF) antibodies to *M. pneumoniae* or the presence of cold agglutinins to aid in diagnosing this infection (6). During the 1980s, sensitive immunoglobulin M (IgM)-specific immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA) procedures began to replace the more cumbersome CF assay and the nonspecific and insensitive cold agglutinin procedure. Both IgM-specific IFA and ELISA procedures reduced the need for paired specimens to make a more accurate diagnosis (12). The IFA is technically simple but is subjective, and the presence of *M. pneumoniae*-specific IgG may interfere with results (3). Thus, IgM must be separated from IgG for accurate results. The *M. pneumoniae*-specific IgM ELISA is objective but is time-consuming, requires complex calculations, and is most cost-effective when specimens are batched.

The ImmunoCard *Mycoplasma* kit from Meridian Diagnostics (Cincinnati, Ohio) is a new, rapid (10 min) enzyme immunoassay card assay designed to detect IgM antibodies to *M. pneumoniae*. This procedure is simple to perform, does not require IgM separation or IgG removal from serum prior to analysis, and would be cost-effective when performed on single specimens or in small batches. We compared the ImmunoCard *Mycoplasma* assay with the INCSTAR *M. pneumoniae* IgM-specific plate ELISA, with the Zeus IgM-specific IFA, and with a standard CF procedure (14). In this paper, we present results of the evaluation and comment on the overall effectiveness and utility of the ImmunoCard *Mycoplasma* assay.

MATERIALS AND METHODS

Specimens. Five sites participated in this project: Summa Health System in Akron, Ohio; Indiana University School of Medicine in Indianapolis; Bethesda Hospitals in Cincinnati, Ohio; Meridian Diagnostics; and Corning Nichols Institute in San Juan Capistrano, Calif. The centers analyzed 896 serum specimens obtained from pediatric or adult patients being evaluated for a presentation consistent with *M. pneumoniae* infection. All specimens were stored refrigerated or frozen (−20°C) prior to testing. Specimens were not freeze-thawed more than once, and no differences in results between frozen or fresh specimens were observed.

ImmunoCard *Mycoplasma* kit. The ImmunoCard *Mycoplasma* procedure (Meridian Diagnostics) was performed according to the manufacturer's directions. This test system consists of a plastic card which contains an absorbent filter paper. The top surface of the card has four openings which provide access to the filter paper. The two bottom openings are serum application ports. The paper in the top right-hand port is impregnated with a *M. pneumoniae* extract (test well), and the paper in the top left-hand port contains a human IgM reagent (control well). Serum was added to the two lower application ports and allowed to migrate to the control and test impregnated regions of the filter. An anti-human IgM alkaline phosphatase conjugate was added to the lower control and test application ports and allowed to react for 2 min. The upper control and test ports were then washed with a buffer supplied in the kit. A substrate solution was then added to the upper wells, and the device was incubated for 5 min. A positive reaction was indicated by development of a definite blue color in the test well.

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TABLE 1. Performance of ImmunoCard *Mycoplasma* assay versus IFA[a]

IC result	IFA result			Resolved by chart review		
	Pos	Neg	Eq	Pos	Neg	NR
Pos	9	10	3	11	3	8
Neg	2	152	3	2	154	1

[a] Abbreviations: IC, ImmunoCard *Mycoplasma* assay; Pos, positive; Neg, negative; Eq, equivocal; NR, not resolved. $n = 179$. ImmunoCard *Mycoplasma* performance versus resolved results: sensitivity, 84.6%; specificity, 98.1%; positive predictive value, 78.6%; negative predictive value, 98.7%; agreement, 97.1%.

TABLE 2. Performance of ImmunoCard *Mycoplasma* assay versus CF[a]

IC result	CF result			Resolved by IFA		
	Pos (>32)	Neg (<8)	Eq (8–32)	Pos	Neg	NR
Pos	37	38	47	80	16	26
Neg	5	141	67	3	150	60

[a] Abbreviations: IC, ImmunoCard *Mycoplasma* assay; Pos, positive; Neg, negative; Eq, equivocal; NR, not resolved. Numbers in parentheses are CF titers. $n = 335$. ImmunoCard *Mycoplasma* performance versus resolved results: sensitivity, 96.4%; specificity, 90.4%; positive predictive value, 83.3%; negative predictive value, 98.0%; agreement, 92.4%.

The control well stained blue to show that the test had been performed properly. Only undiluted, unfractionated serum was used. Specimens which showed a blue color in the test well were recorded as positive; specimens without any blue color were considered negative. Any specimen with no blue color in the control port was considered invalid.

IFA. The *Mycoplasma* IgM Crowntiter Assay (Zeus, Inc., Raritan, N.J.) was used at Summa Health System in comparison studies according to the manufacturer's directions. IgG was removed from all sera by the Isolab Quick Sep System (Isolab, Akron, Ohio), and the resulting IgM-containing fractions were assayed at final 1:16 and 1:32 dilutions. Specimens reactive at the lower (1:16) dilution only were reported as equivocal, and those reactive at the higher (1:32) dilution were considered positive for IgM anti-*M. pneumoniae*, in accordance with the manufacturer's recommendations. The Zeus standard IgM IFA was used at all other sites to aid in resolving equivocal specimens. According to manufacturer's instructions, specimens assayed on the standard IFA were diluted to 1:8 and 1:16. In the standard procedure, a reactive result at 1:8 is considered equivocal and a reactive result of 1:16 is considered positive.

ELISA. A plate *Mycoplasma* IgM ELISA (INCSTAR, Inc., Stillwater, Minn.) was used in direct comparison studies with the ImmunoCard *Mycoplasma* assay. This is an IgM capture assay. Patient serum was incubated on an anti-IgM-coated plate. The plate was then washed, and an antigen conjugate was added. Following substrate addition, optical density at 405 nm was determined on a microplate reader. Cutoff and quality control values were calculated as per the manufacturer's package insert. Specimens which generated absorbance values below the cutoff were considered negative, those above were positive, and those which fell into the retest range, as defined by the manufacturer, were classified as equivocal. Specimens designated as equivocal were assayed on the Zeus standard IFA.

Complement fixation. Specimens were assayed by a standard Laboratory Branch CF procedure (14) with a *Mycoplasma* antigen preparation obtained from Bio Whittaker Diagnostics (Walkersville, Md.). Each specimen was tested in twofold dilutions from 1:8 to 1:8,192. A titer of 64 or higher was considered as positive and consistent with active disease. Titers of 8 to 32 were considered equivocal.

Statistical analysis. The sensitivity, specificity, and assay correlations were calculated for the ImmunoCard *Mycoplasma* kit with the comparison method (CF, ELISA, or IFA) as a reference standard. No "gold standard" exists for *M. pneumoniae* serology; thus we defined a true result for specimens which had equivocal or retest interpretations by testing by a third method or by clinical chart review. The result obtained by the third method or chart review was listed as a resolved result. We then compared the ImmunoCard results with resolved results. The tables indicate when resolved results were used in the calculations.

Patient chart reviews. Where possible, we reviewed medical records of individuals who tested positive or equivocal by one or more assays. We classified patients as infected with *M. pneumoniae* if they met one of the following criteria: (i) a physician-documented clinical diagnosis of *M. pneumoniae* infection, based upon clinical symptoms and/or serological assays; and (ii) in the absence of a definitive diagnosis, the presence of symptoms consistent with *M. pneumoniae* infection, such as a nonproductive cough, fever, anemia, and response to treatment, plus at least one positive IgM serological assay (excluding the ImmunoCard assay). Patients with no clear *M. pneumoniae* symptoms or patients who had a clinical diagnosis other than *M. pneumoniae* were classified as not infected.

RESULTS

ImmunoCard *Mycoplasma* assay versus IFA. We analyzed 179 specimens by both the ImmunoCard assay and IFA at Summa Health System. Results are shown in Table 1. The two tests agreed on 93.1% of the specimens. The IFA was able to classify 173 specimens as either positive or negative, resulting in an equivocal rate of 3.4%. All ImmunoCard *Mycoplasma* results were read as positive or negative. To resolve the 18 discrepant or IFA-equivocal specimens, we reviewed patients' charts for all specimens which were positive or equivocal by one or both assays. This procedure resolved nine of the discrepant specimens, resulting in an overall agreement of 97.1%. Chart data were not helpful in resolving the remaining nine of the discrepant or IFA-equivocal specimens. The resolved ImmunoCard *Mycoplasma* sensitivity, specificity, and agreement were 84.6, 98.1, and 97.1%, respectively. Complete performance statistics for the ImmunoCard *Mycoplasma* assay compared with these clinically resolved results are shown in Table 1.

ImmunoCard *Mycoplasma* assay versus CF. We compared results from 335 specimens (137 at Indiana University and 198 at Corning Nichols Institute) tested by both the ImmunoCard *Mycoplasma* assay and CF. Results are shown in Table 2. The two tests agreed on 80.5% of the 221 specimens classified as positive or negative by CF. CF classified 114 specimens (34%) as equivocal (titers of 8 to 32). The ImmunoCard *Mycoplasma* method was positive for 47 (41%) of the CF-equivocal specimens and was negative for the remaining CF-equivocal 67 (59%). Figure 1 shows the CF titer results plotted against the percent positive by ImmunoCard. This figure shows that ImmunoCard *Mycoplasma* has 79% sensitivity at the lowest positive CF titer of 64 and reaches 100% sensitivity at CF titers of ≥256. We were unable to use patient chart review to resolve the discrepant or CF-equivocal specimens' results. The majority of these specimens were submitted to a reference laboratory

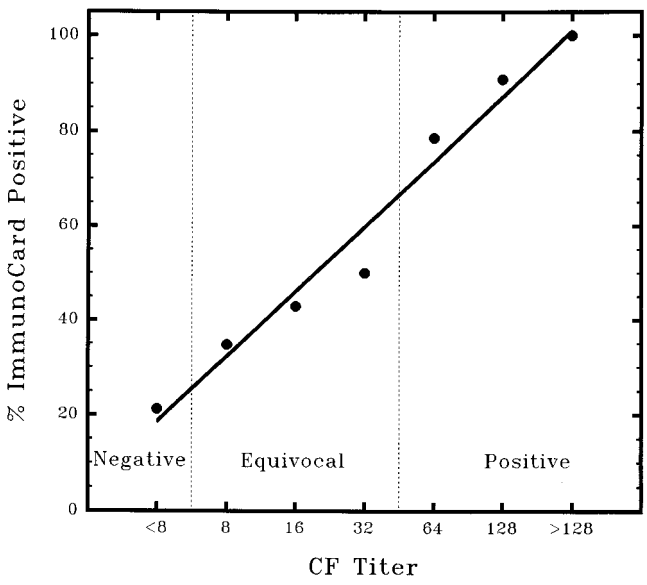

FIG. 1. Percentage of specimens which were ImmunoCard *Mycoplasma* assay positive at each complement fixation titer. The appropriate CF interpretation is listed for each range of titers.

TABLE 3. Performance of ImmunoCard *Mycoplasma* assay versus ELISA[a]

IC result	ELISA result			Resolved by IFA		
	Pos	Neg	Retest	Pos	Neg	NR
Pos	143	89	30	216	22	24
Neg	51	516	27	14	563	17

[a] Abbreviations: IC, ImmunoCard *Mycoplasma* assay; Pos, positive; Neg, negative; NR, not resolved. $n = 856$. ImmunoCard *Mycoplasma* performance versus resolved results: sensitivity, 93.9%; specificity, 96.2%; positive predictive value, 90.8%; negative predictive value, 97.6%; agreement, 95.6%.

(Nichols), and thus patient charts were not available for review. We had sufficient volume to assay 76 of the 157 (48%) discrepant and CF-equivocal specimens by the Zeus standard *M. pneumoniae* IgM IFA. Results from these assays are presented in Table 2. A resolved result was defined as positive if the IFA was positive. A resolved result is defined as negative if the IFA was negative. All other combinations are treated as unresolved. These resolved results demonstrate 96.4% sensitivity, 90.4% specificity, and 92.4% agreement for the ImmunoCard *Mycoplasma* assay. This analysis was unable to resolve 86 specimens.

ImmunoCard *Mycoplasma* assay versus ELISA. We compared 856 specimens (92 at Bethesda Hospital, 219 at Meridian Diagnostics, 366 at Nichols Institute, and 179 at Summa Health System) by both the ImmunoCard and the INCSTAR ELISA procedures. Overall ELISA comparison results are presented in Table 3. The ImmunoCard demonstrated an 82.5% correlation with the ELISA. The ELISA initially classified 57 (6.7%) specimens as retest. These 57 specimens, along with those which had discrepant results between the ELISA and ImmunoCard *Mycoplasma* assay, were subjected to IFA testing for resolution. A positive IFA test along with a positive or retest ELISA test was considered a positive resolved result. A negative IFA test along with a negative or retest ELISA result was considered negative. All other combinations were classified as unresolved. Results of this analysis are presented in Table 3. These data show a 95.6% agreement between the resolved results and the ImmunoCard assay. The ImmunoCard *Mycoplasma* sensitivity was 93.9%, and the specificity was 96.2% in this comparison.

ImmunoCard *Mycoplasma* assay versus consensus results. As a final comparison, we devised a more complete truth table for the study (Table 4). We evaluated 694 specimens by at least two other tests in addition to the ImmunoCard *Mycoplasma* assay. We defined a true-positive specimen as one which was positive in two or more of the IFA, ELISA, CF, or chart review analyses. We then compared each of the assays with these final resolved results. The data are shown in Table 4. The ImmunoCard had the highest sensitivity (90%) of all of the assays. The ImmunoCard's specificity was the lowest of the assays, although it was still 93%. Overall, the IFA had the best correlation (96%), followed by the ImmunoCard (92%). The CF assay had the poorest correlation (81%). This was to be expected because the CF assay was the only non-IgM-specific procedure evaluated.

DISCUSSION

We compared the Meridian ImmunoCard *Mycoplasma* assay with IFA, ELISA, and CF for the detection of *M. pneumoniae*-specific antibodies in 896 clinical specimens. Overall, the ImmunoCard *Mycoplasma* assay performed well in this evaluation, showing an overall correlation of 92% (Table 4). The interpretation of the comparisons is difficult because there is no gold standard for *Mycoplasma* IgM testing. Although the IFA and ELISA are IgM-specific assays, published reports do not show them to be 100% accurate (10). The CF assay has been used as a standard for *Mycoplasma* serologic testing, although questions remain as to its clinical interpretation (8). The CF test detects IgG antibodies in addition to IgM antibodies, and so a direct comparison of results obtained from the IgM-specific method and CF would not be expected to yield a 100% correlation. Standard performance statistics are also difficult to use to compare assays which report results in three categories (positive, negative, and equivocal) with assays which report results in two categories (positive and negative).

The ImmunoCard-with-IFA comparison had eight ImmunoCard-positive specimens for which no agreement between the methods could be obtained. The clinical status of those eight individuals could not be reliably determined. The ImmunoCard *Mycoplasma* assay uses unfractionated, undiluted serum and thus may detect low levels of anti-*Mycoplasma* IgM which are diluted out in the IFA procedure. These clinically unresolved specimens may represent biologic false-positive results or may indicate that the ImmunoCard *Mycoplasma* assay detects lower levels of antibody than the IFA. Three of the eight unresolved specimens were positive, two were equivocal, and three were negative when tested by the INCSTAR ELISA method (data not shown). Final resolution was, again, hampered by the lack of a standard procedure.

The low sensitivity observed for ImmunoCard results compared with low-titer CF specimens (Fig. 1) may be due to the fact that some specimens may have been negative for IgM anti-*M. pneumoniae* antibodies but positive for IgG anti-*M. pneumoniae* antibodies. These specimens would have a predicted reactivity of negative in the ImmunoCard *Mycoplasma* test and positive in the CF assay. IgG is not as potent a CF antibody as is IgM (1), thus IgG-positive, IgM-negative specimens may be expected to have low CF titers. These low-titer CF-positive specimens which were ImmunoCard negative would reduce the statistical sensitivity of the ImmunoCard *Mycoplasma* assay; this is what we observed. The ImmunoCard results showed high sensitivity compared with CF results resolved with the addition of an IgM-specific IFA, supporting the

TABLE 4. Performance of each assay versus consensus results

Method	Result[a]	No. of true positives[b] ($n = 174$)[c]	No. of true negatives[b] ($n = 520$)[c]	Sensitivity (%)	Specificity (%)	Agreement (%)
ImmunoCard	Pos	156	35	90	93	92
	Neg	18	484			
	Invalid	0	0			
ELISA	Pos	100	24	68	95	88
	Neg	47	422			
	Retest	13	32			
IFA	Pos	86	2	89	99	96
	Neg	11	209			
	Eq	5	11			
CF	Pos (>32)	39	3	51	98	81
	Neg (<8)	38	141			
	Eq (5–32)	40	83			

[a] Pos, positive; Neg, negative; Eq, equivocal. Numbers in parentheses are CF titers.

[b] True positives are specimens which tested positive on two or more assays other than ImmunoCard. All other result combinations are treated as true negatives.

[c] Total number of specimens tested in two or more assays. The *n* for each individual assay varies.

hypothesis that the ImmunoCard may also be empirically more sensitive than CF for detecting IgM.

The ImmunoCard *Mycoplasma* assay results showed the lowest sensitivity compared with results obtained by the ELISA. Resolving the ELISA results with a second IgM-specific assay, the IFA, improved the ImmunoCard statistics to over 90% for each parameter (Table 3). Neither the ELISA nor the IFA may be considered a gold standard (10); thus the combination of the results may be the most useful comparison. Their respective package inserts show sensitivities of 82.3 and 77.3% and specificities of 85.6 and 97.6% compared with CF. One site (Summa) assayed 179 specimens by both the ELISA and the Crowntiter IFA and found a 93% correlation between the methods but only a 40% IFA sensitivity compared with that of the ELISA (data not shown). Whether this represents low sensitivity on the part of the IFA or possible false positives on the part of the ELISA was not addressed in this study.

The ImmunoCard *Mycoplasma* assay can be performed in less than 10 min, and batches of up to 10 specimens may be run concurrently. Reading the blue color on the ImmunoCard *Mycoplasma* assay may take some practice; however, we judged (very subjectively) the ImmunoCard easier to read (overall) than the IFA slides. The package insert states that any visually detectable blue color covering the entire test and control ports is indicative of a positive result, and no equivocal result is described. Blue color over just one side of the test port is interpreted as a negative reaction. We did not see any evidence of partially colored ports in our study. Replicate studies commissioned by Meridian Diagnostics found 95% agreement on reading the blue color among replicate specimens sent to three different physician office laboratories (16).

The IFA takes approximately 90 min to perform and requires a fluorescent microscope and a well-trained individual to read the slides. The ELISA is read objectively, takes up to 4 h to perform, and requires a plate reader and complex calculations (which may be automated). In both the IFA and ELISA, large batches of specimens may be run easily. The CF procedure requires an overnight incubation and is most cost-effective (and technically demanding) when large numbers of specimens are run in a batch. Of interest, Thacker and Talkington (13) compared CF with two rapid *M. pneumoniae* antibody kits designed to diagnose current infection. They found that, although the tests were technically easier than CF, one test had lower sensitivity and the other required paired specimens for most effective use. Neither of the kits included in the Thacker and Talkington study was IgM specific (13), and thus we did not include them in our comparisons.

In conclusion, the ImmunoCard *Mycoplasma* assay is a valid methodology for detecting IgM antibodies to *M. pneumoniae*. This procedure can decrease turnaround time and technologist time compared with IFA, ELISA, or CF and thus should reduce labor costs for laboratories assaying small numbers of specimens at a time. The ImmunoCard achieved its highest performance statistics against the resolved or consensus results, a fact that emphasizes its utility, even in the absence of a gold standard. The ImmunoCard *Mycoplasma* assay joins a growing trend in adapting ELISA technology to rapid, simple assays which perform well in a clinical setting (9, 11, 13, 15). Our comparisons indicate that the ImmunoCard *Mycoplasma* assay is appropriate for use in a hospital laboratory to provide rapid testing of patients presenting with atypical pneumonia symptoms.

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