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Research paper

Antineutrophil cytoplasmic antibody (ANCA) testing of routine sera varies in different laboratories but concordance is greater for cytoplasmic fluorescence (C-ANCA) and myeloperoxidase specificity (MPO-ANCA)

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ABSTRACT

Most laboratories screen for antineutrophil cytoplasmic antibodies (ANCA) with indirect immunofluorescence (IIF) and confirm cytoplasmic (C-ANCA) and perinuclear (P-ANCA) staining with ELISAs for proteinase 3 (PR3) and myeloperoxidase (MPO) specificities. This study determined the concordance of ANCA test results from 48 diagnostic laboratories participating in a national Quality Assurance programme, that used different assays and methods and varied in expertise. Laboratories were circulated with a questionnaire about their techniques, and provided with 24 sera for testing over a 30 month period. Results for individual sera were compared with the 'observed consensus' found in more than 50% of laboratories. The 23 laboratories (48%) that responded to the questionnaire used 5 different IIF substrates and 11 ELISAs, and differed in other aspects of testing. Concordance for ANCA test results was greater for IIF-positive ($n=22$, median 96%, range 68%–100%) than an IIF-negative serum (median 64%); for C-ANCA ($n=8$, median 89%, range 66–100%) rather than P-ANCA ($n=10$, median 76%, range 52–88%); for MPO-ANCA ($n=5$, median 100%) rather than PR3-ANCA ($n=7$, median 89%, range 82–100%); and for strongly-positive ($n=2$, median 97%, range 96–97%) rather than low positive PR3-ANCA ($n=4$, median 80%, range 74–86%). Concordance for test results might be improved with further standardisation of testing methodologies.

1. Introduction

Antineutrophil cytoplasmic antibodies (ANCA) are autoantibodies directed against neutrophil cytoplasmic enzymes and are useful in the diagnosis and management of systemic small vessel vasculitis (Davies et al., 1982; van der Woude et al., 1985; Savige et al., 2000). Most laboratories screen for ANCA using indirect immunofluorescence (IIF) which results in cytoplasmic (C-ANCA) or perinuclear (P-ANCA) patterns. They then confirm antibody specificity in ELISAs for proteinase 3 (PR3) and myeloperoxidase (MPO) specificities (Goldschmeding et al., 1989; Falk and Jennette, 1988). C-ANCA directed against PR3 occur in 70% of patients with limited Wegener granulomatosis and 90% of those with generalised disease. P-ANCA directed against MPO are found in 80% of patients with microscopic polyangiitis, renal-limited pauciimmune segmental necrotising glomerulonephritis, and the 'microscopic polyangiitis/polyarteritis nodosa' syndrome.

Variation in ANCA test results from different laboratories occurs because of differences in assays, laboratory protocols and expertise. This variation is well-described (Trevisin et al., 2008). The 'International Consensus Statement on Testing and Reporting ANCA' was developed to encourage more uniformity in ANCA testing protocols and reporting (Savige et al., 1999, 2003).

The Royal Australasian College of Pathologists of Australasia (RCPA) is the national authority for quality assurance (QA) for the disciplines of pathology in Australia and New Zealand, and provides an equivalent programme to those in the United Kingdom (National External Quality Assessment

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Scheme, UKNEQAS) and the United States (College of American Pathologists, CAP). The study described here investigated the variation in ANCA testing methods and determined which aspects of the ANCA result were most robust despite this variation.

2. Methods

2.1. Survey of laboratories taking part in the QA programme

Laboratories participating in the RCPA QA programme were asked to complete a detailed questionnaire indicating the ANCA assays and reagents they were asked about: the use of both PR3- and MPO-ANCA ELISAs to confirm IIF-positivity; testing on formalin-fixed neutrophils; serum dilutions; the source of conjugates; optimization of conjugate dilutions; magnification for IIF; permissible delay before reporting IIF patterns; the source of ELISA validation data; how calibration curves were constructed; the use of non-kit controls; how background was corrected for; the terms used for reporting; and whether semiquantitative or qualitative data were reported.

2.2. RCPA QA programme for ANCA testing

Twenty-four sera were sent to a maximum of 48 laboratories over a 30 month period with instructions for storage and thawing. Sera for testing were provided by laboratories participating in the QA programme and were usually chosen to present routine but not exceptional challenges in reporting.

Laboratories tested individual sera at designated times throughout the year in their routine assays, and submitted the results by specified dates to the QA programme co-ordinator. The results were provided anonymously to all participating laboratories.

2.2.1. Analysis of test results

Since there was no 'gold standard' for ANCA results, the QA programme designated any result 'positive' if it were found by at least 50% of the testing laboratories. The QA programme then used two methods to determine satisfactory performance. These were the % laboratories that reported the majority result ('observed' consensus) and the 'true' consensus when this number reached 95% confidence limits ($p<0.05$). For the 'true' consensus the maximum number of laboratories that could differ from the majority result depended on the total number participating (ISO/IEC GUIDE, 1997). This number was 0 for 25 laboratories, 1 for 26–33, 2 for 34–40 and 3 for 41–47.

3. Results

3.1. Variation in assays and protocols for ANCA testing and reporting

Twenty-three of the 48 laboratories (48%) participating in the QA programme returned completed questionnaires. These demonstrated substantial variation in ANCA testing assays and methodologies. All 23 screened for ANCA by IIF. Four (17%) performed IIF plus ELISAs for both PR3 and MPO-ANCA on all sera; 16 (70%) confirmed IIF-positive sera in both PR3- and MPO-ANCA ELISAs; 2 (9%) confirmed C-ANCA and P-ANCA only with the PR3- and MPO-ANCA ELISAs respectively; and one laboratory (4%) tested only by IIF.

3.1.1. IIF testing

The laboratories used 5 different neutrophil substrates, 4 commercial (22/23, 96%) and one in-house preparation. The commercial preparations were from INOVA (INOVA Diagnostics Inc, CA, USA) (15, 65%), ImmunoConcepts CA, USA (3, 13%), EUROIMMUN, Seekamp, Germany (3, 13%), and The Binding Site, Birmingham, UK (1, 4%). All neutrophil preparations were ethanol-fixed, but 3 laboratories also tested on formalin-fixed cells (3, 13%, EUROIMMUN). Laboratories used 5 different serum dilutions for IIF (1/20 (13, 57%), 1/10 (13, 57%), 1/16 (1, 4%) 1/30 (1, 4%) and 1/40 (4, 17%)). Sera were diluted in phosphate-buffered saline (PBS, 18/23, 78%) or PBS with bovine serum albumin (5, 22%). Six different conjugates were used, with 21 laboratories (92%) using antiIgG and 2 (9%) antiIgG, A and M. Laboratories that tested with ImmunoConcepts and EUROIMMUN slides used the conjugates provided by the manufacturers and 11 (73%) of the 15 INOVA users did. The remaining 6 laboratories (26%) tested with conjugates from other manufacturers and optimised them in-house by checkerboard titration.

Thirteen laboratories (57%) examined the IIF preparations with a final magnification of ×400 and 9 (39%)×200. All laboratories examined the IIF and interpreted the patterns within 4 h, and 6 (6/20, 30%) described finding differences in IIF if slides were re-examined the following day. In these cases the IIF was less intense, patterns became more diffuse, and P-ANCA more closely resembled C-ANCA or 'atypical' ANCA. These changes were not specific to preparations from any manufacturer.

3.1.2. PR3 and MPO-ANCA testing

Nineteen laboratories tested with 11 different ELISAs including 2 in-house methods and 1 enzyme fluoroimmunoassay. Most laboratories that used commercial ELISAs (14/17, 82%) obtained both PR3-ANCA and MPO-ANCA assays from the same manufacturer.

For PR3-ANCA, 8 laboratories (42%) tested with the ORGENTEC assay (ORGENTEC Diagnostika GmbH, Mainz, Germany); 2 (11%) each used Eurodiagnostica (Malmo, Sweden) or SCIMEDEX (SCIMEDX Corporation, New Jersey, USA); and one each used (5%) assays from Bindazyme, Kallestad (Bio-Rad Laboratories, California, USA), INOVA, Biodiagnostica Rainbow ELISA (Bio-Diagnostics Ltd, Worcestershire, UK), and the EliA (Pharmacia Diagnostics, Freiberg, Germany).

For MPO-ANCA, 11 laboratories (58%) tested with the Orgentec assay, and each (5%) of the assays from Eurodiagnostica, SCIMEDX, Bindazyme, Kallestad, INOVA Diagnostics, Biodiagnostica, and Pharmacia.

Eight laboratories (40%) used the manufacturer's validation data (sensitivity, specificity, precision and linearity) as well as their own, 8 (40%) used only the manufacturers' data, and 4 laboratories (20%) only their own.

Eighteen laboratories (95%) derived calibration curves from multiple points and one (5%) from a single point (INOVA assay). Ten laboratories (53%) performed the assay in duplicate and a further laboratory tested the calibrators in