Technical reports

Histological identification of carcinoma in 21 gauge needle tracks after fine needle aspiration biopsy of head and neck carcinoma

A J Mighell, A S High

Abstract
Six cancer resection specimens were thoroughly sectioned and microscopically examined at areas known to have been around 21 gauge fine needle aspiration (FNA) biopsy sites, in an attempt to identify needle tracks. All cases had an interval of not less than 10 days between FNA biopsy and surgery. Foci of tumour were identified histologically in needle tracks from two patients with carcinoma. This is the first instance, outside of experimental animal models, of histologically confirmed, viable tumour spread in FNA biopsy tracks. Although this complication is not common and is of unknown clinical significance, it is one that all clinicians who undertake FNA of malignant neoplasms should be aware of.
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In many instances, establishment of a tissue diagnosis is fundamental to the implementation of appropriate clinical management. Tumour biopsy morbidity is dependent on biopsy technique, lesion location, and pathological tissue type. Fine needle aspiration (FNA) biopsy is an established technique associated with minimal morbidity compared with more invasive biopsy techniques such as wide bore needle biopsy or open biopsy.[1–3] However, tumour seeding is recognised as a rare complication of FNA biopsy, particularly of abdominal and thoracic neoplasms.[4]

The chance finding of an apparent needle track with an associated small focus of carcinoma in a wide excision specimen led us to investigate a further five cases where FNA biopsy had preceded tumour excision. Microscopic examination of multiple blocks and sections were done in an attempt to identify needle tracks.

Methods
Cancer resection specimens from six patients with malignant tumours were examined for evidence of needle related damage or tumour spread around sites known to have had needle biopsies. Multiple blocks were taken from regions known to have been crossed by needles and these blocks were serial sectioned at 3 µm.

All patients had had a previous FNA biopsy using a 21 gauge needle. The interval between FNA biopsy and surgery was at least 10 days (mean (SD) 21.3 (8.9) days).

Results
Needle tracks were identified in two of the six patients, and both cases demonstrated apparent tumour dissemination along those tracks.

CASE 1
A 50 year old white man presented with a three year history of a slow growing palatal mass. Extra-oral examination was unremarkable with no clinical lymphadenopathy identified. Intra-orally, a 5 × 3 cm exophytic, purple, firm swelling with localised areas of ulceration involved the left hard and soft palate. The lesion was defined clinically with no involvement of either the pharyngeal wall, posterior nasal space or nasal lining. There was no sensory or motor deficit of either the trigeminal or glossopharyngeal nerves. Occipitomental radiographs, axial computed tomography, and chest radiography did not identify more extensive disease. An incision biopsy of the palatal lesion was difficult to interpret but was suggestive of adenoid cystic carcinoma. Under general anaesthesia a left hemimaxillectomy was done via a modified Weber-Ferguson incision, mandibular lip split, and mandibulotomy. The lesion was circumscribed with a thin, poorly formed capsule in places. The resected specimen included the left maxilla, soft palate with uvula, floor of nose, and lateral wall of the pharynx. During surgery, a small, firm, enlarged lymph node was palpated in the left submandibular triangle. FNA biopsy of this lymph node was undertaken via the floor of the mouth with three to four passes of a 21 gauge needle (Green, 21G2, 0.8 × 50 mm, Microlance 3; Becton Dickinson, Dublin, Ireland) attached to a 10 ml syringe.

Histology of the main specimen revealed a circumscribed, salivary gland neoplasm, with localised areas of invasion of adjacent structures. The neoplasm comprised cords and solid

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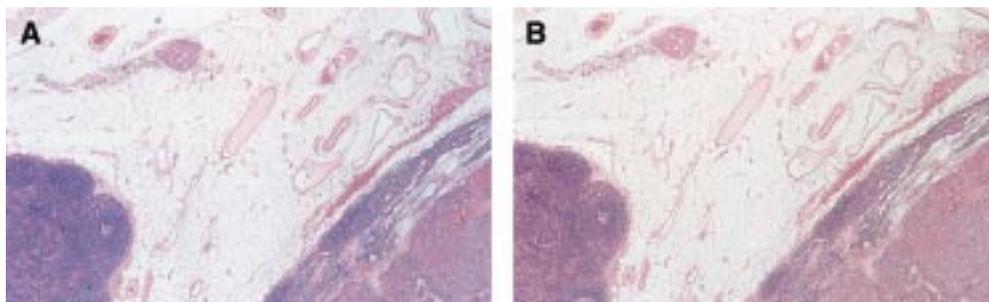A J Mighell
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Figure 1   (A) A focus of adenocarcinoma (mid-left) is observed in the loose connective tissue between a normal lymph node (bottom left) and a lymph node centrally replaced by tumour (right) in the absence of extracapsular tumour spread. The tumour focus lies at one end of a track of multinucleated giant cells and mononuclear inflammatory cells (haematoxylin and eosin, original magnification ×25). (B) The same field as (A) viewed through crossed polarisers. Optically active material is evident along the length of the track, associated with giant cells (original magnification ×25).
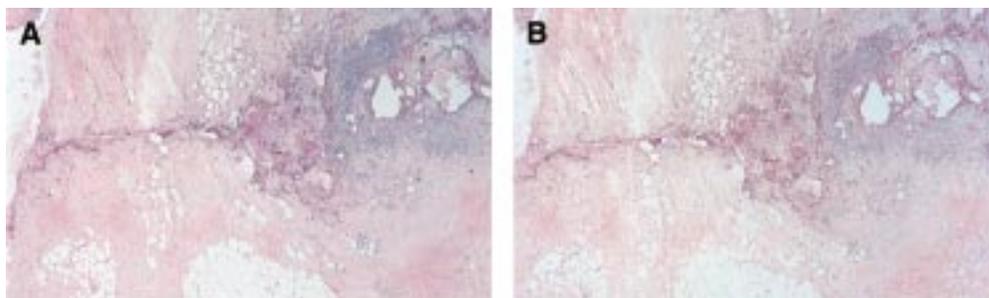

Figure 2   (A) A deposit of squamous cell carcinoma is identified just off the figure on the right margin, with a track of multinucleated, foreign body-type giant cells, and mononuclear inflammatory cells seen extending from this to the margin of the block. Within this track, small foci of tumour are noted (haematoxylin and eosin, original magnification ×25). (B) The same field as (A) viewed through crossed polarisers. Scanty, optically active material is evident along the length of the track (original magnification ×25).

islands of cells with indistinct cell membranes and large, cytologically bland, pale staining vesicular nuclei. There were approximately three mitoses per high power field. There was minimal cellular pleomorphism. Areas of tumour were separated by trabeculae of fibrous tissue, and localised areas of either marked vascularity or tumour necrosis were identified. No perineural spread was observed, and all margins were clear of tumour. A diagnosis of adenocarcinoma (NOS) was made. Cytology of the FNA biopsy tissue identified malignant cells.

Thirty three days after the initial resection, a left radical neck dissection was done. Histological assessment of a 2.5 × 1.5 × 1.0 cm submandibular lymph node identified partial replacement by adenocarcinoma of similar histology to the palatal neoplasm. A single, small focus of malignant cells was observed between this lymph node and the adjacent normal submandibular gland (fig 1A). This focus was at the end of a track of inflammatory cells, and multinucleated foreign body-type giant cells, some of which contained optically active, foreign material, presumably derived from surgical gloves (fig 1B). These appearances were interpreted as implantation of adenocarcinoma along the FNA biopsy needle track. Examination of further histological levels failed to identify either further foci of malignant cells or extracapsular spread from the involved lymph node. Fourteen other cervical lymph nodes from the neck dissection were examined; no further neoplasia was identified.

Seven months after left neck dissection, right submandibular and jugulodigastric lymphad-

enopathy was identified. The patient refused further surgery, and palliative radiotherapy was given. He died 10 months later with widespread adenocarcinoma.

CASE 2
An 85 year old man had had a wedge excision of lower lip for a crusted lesion that proved to be a moderately differentiated squamous cell carcinoma. Margins appeared clear histologically and no further treatment was given. Thirty months later he presented with a submandibular mass. This was thought to be either lymphadenopathy or an enlarged submandibular salivary gland. FNA biopsy was performed via the skin, with three to four passes of a 21 gauge needle (Green, 21G2, 0.8 × 50 mm) attached to a 20 ml syringe; however, results were largely haemorrhagic, with only a few squamous elements present. These were not felt to be profoundly atypical, but in view of their sparsity the final report was inconclusive. The aspirate was reported as haemorrhagic with a little supporting connective tissue, but with no evidence to support a diagnosis of recurrent carcinoma. He was given antibiotics and reviewed at a later appointment. It became apparent that the lesion was enlarging, so he was admitted for neck dissection. A radical neck dissection including submandibular gland was performed 29 days after FNA biopsy. Histological assessment of the submandibular gland revealed well differentiated squamous cell carcinoma. No convincing lymph node was identified, but this was felt likely to represent extensive replacement and obliteration following metastatic spread to

one or more submandibular lymph nodes. The adjacent submandibular gland was unremarkable. At one margin of the specimen, however, a clear track of inflammatory cells (fig 2A) and optically active material (fig 2B) was identified. Along its length, small islands of carcinoma were identifiable (fig 2A). Surgery was followed by local radiotherapy to the neck. The patient is currently well, with no evidence of residual disease.

Discussion

Dissemination of malignant cells is a risk of any cancer biopsy. FNA biopsy is a minimally invasive procedure with low rates of morbidity and mortality compared with more invasive biopsy procedures such as either wide bore needle biopsy or open biopsy.[1-4] Clinically detectable metastases that can be attributed to FNA biopsy are typically located to skin puncture wounds, and are detectable in fewer than 0.009% of cases.[4] Although few clinical metastases can be attributed to FNA biopsy, animal studies indicate that dissemination of malignant cells frequently follows FNA biopsy. FNA biopsy with an 18 gauge needle of rabbit popliteal lymph nodes containing metastatic squamous cell carcinoma was associated with local spread of carcinoma cells outside the lymph node capsule in 10 of 12 rabbits.[5] FNA biopsy, with a 0.6 mm diameter needle, of solid murine tumours through healthy leg muscle, was associated with tumour growth around the needle track in 65–83% of mice.[6] In the same study, 0.6 mm needle FNA biopsy of radiolabelled murine ascitic tumours was undertaken through healthy leg muscle. Measurement of radioactivity suggested that 10^3–10^4 tumour cells may be seeded into mouse leg muscle as a result of FNA biopsy. However, the authors state that these studies must be interpreted with caution as the experimental tumour cell lines investigated have an aggressive phenotype, and contain few mesenchymal or inflammatory cells.[6] Using a different approach, FNA biopsy was done immediately after resection of 40 specimens of human lung cancer.[7] Malignant cells were identified histologically in 89% of needle tracks through normal lung after FNA biopsy with a 1.0 mm diameter needle. Although FNA biopsy may spread tumour cells along the needle track, few malignant cells probably survive. Some tumour cells will be

physically damaged, others will be non-viable outside the main tumour mass, and the host immune system probably destroys most malignant cells disseminated from the main tumour mass by FNA biopsy. Finally, many potential tumour seedlings are excised with the main specimen at definitive surgery. An investigation of FNA biopsy, incisional and excisional biopsy of experimental murine tumours concluded that there was little difference in death rates between the study groups.[8] This is supported by large, retrospective studies in humans, where clinically significant tumour cell dissemination can rarely be attributed to FNA biopsy.[4] Accordingly, the behaviour of the carcinomas in the two cases presented is consistent with an aggressive malignant phenotype, particularly as the foci of malignant cells were probably a consequence of FNA biopsy 33 and 29 days earlier.

CONCLUSION
- Dissemination of malignant cells during tumour biopsy is probably not uncommon, but rarely results in clinically identifiable metastases.
- The cases presented illustrate foci of malignant cells implanted during FNA biopsy, and confirm that a minimally invasive biopsy technique can spread viable malignant cells.
- The probability of dissemination appears greater with an aggressive tumour phenotype.

1 Gooder P, Palmer M. Cervical lymph node biopsy—a study of its morbidity. *J Laryngol Otol* 1984;**84**:1031–40.
2 Schwarz R, Chan NH, MacFarlane JK. Fine needle aspiration cytology in the evaluation of head and neck masses. *Am J Surg* 1990;**159**:482–5.
3 Birchall MA, Walsh-Waring GP, Stafford ND. Malignant neck lumps—a measured approach. *Ann Roy Coll Surg Eng* 1991;**73**:91–5.
4 Smith E. Complications of percutaneous abdominal fine needle biopsy. *Radiology* 1991;**178**:253–8.
5 Engzell U, Esposti PL, Rubio C, *et al*. Investigation of tumour spread in connection with aspiration biopsy. *Acta Radiol* 1971;**10**:385–98.
6 Ryd W, Hagmar B, Eriksson O. Local tumour seeding by fine needle aspiration biopsy; a semiquantitative study. *Acta Path Microbiol Immunol Scand Sect A* 1983;**91**:17–21.
7 Struve-Christensen E. Iatrogenic dissemination of tumour cells; dissemination of tumour cells along the needle track after percutaneous, transthoracic lung biopsy. *Dan Med Bull* 1978;**25**:82–7.
8 Eriksson O, Hagmar B, Ryd W. Effects of fine needle aspiration and other biopsy procedures on tumor dissemination in mice. *Cancer* 1984;**54**:73–8.

Evidence of novel pathogenic pathways for the formation of antigastric autoantibodies in *Helicobacter pylori* gastritis

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Abstract
Autoantibodies against gastric epithelial cells are detectable in up to 50% of patients with chronic, active *Helicobacter pylori* gastritis. Presence of autoantibodies against canalicular structures within human parietal cells (anticanalicular autoantibodies) correlate with gastric mucosa atrophy. It has been suggested, that molecular mimicry between *H pylori* and the host on the level of Lewis X and Lewis Y blood group antigens leads to these autoantibodies. This study aimed at analysing whether antigastric antibodies can be absorbed to Lewis X or Y positive *H pylori* strains. Sera from 14 *H pylori* infected patients with anticanalicular autoantibodies were effectively absorbed to *H pylori*. Immunohistochemical studies of the absorbed sera showed no decrease of antigastric autoreactivity. Pathogenic mechanisms other than molecular mimicry lead to the formation of antigastric autoantibodies, and epitopes other than Lewis antigens are the autoimmune targets.
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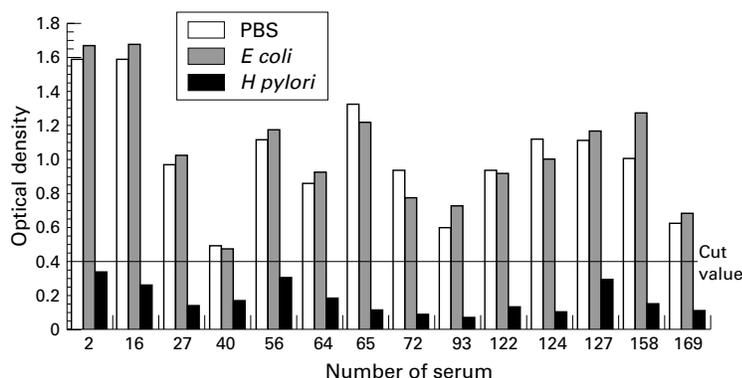Accepted for publication
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Helicobacter pylori infection causes chronic active gastritis and leads to further severe diseases in the upper gastrointestinal tract. It has recently been shown that antigastric autoantibodies to human gastric mucosa occur in about 50% of *H pylori* infected patients.[1–4] In particular, autoantibodies to canalicular structures within parietal cells of the oxyntic mucosa are strongly correlated with *H pylori* infection. This type of antigastric autoreactivity seems to be of particular clinical relevance, as the presence of these autoantibodies is positively correlated with a higher degree of body gastritis, atrophic changes in the gastric mucosa, raised fasting gastrin concentrations, and decreased serum pepsinogen I:II ratio.[4] These findings suggest that the host's autoimmune response in chronic *H pylori* gastritis affects the course of the disease.

Structural chemistry has shown that the lipopolysaccharide of most *H pylori* strains contains the human blood group antigens Lewis X and Lewis Y.[5] As these antigens are also expressed on epithelial cells in the gastric mucosa, and *H pylori* infected mice produce anti-Lewis antibodies, it has been proposed that this molecular mimicry is responsible for the formation of antigastric autoantibodies.[3 6] However, no absorption studies with *H pylori* strains, which are known to be positive for Lewis X or Y, have been performed. This study aimed at investigating whether molecular mimicry between Lewis X and Y positive *H pylori* strains and the host is responsible for the formation of antigastric autoantibodies in *H pylori* gastritis.

Material and methods

Fourteen patients were included in our study. Four gastric biopsy specimens from the antrum and body from each patient were examined histologically. Colonisation of *H pylori* was demonstrated by a Warthin-Starry stain. Sera of all patients were taken shortly before or soon after endoscopy and were screened for IgG antibodies reacting to *H pylori* in an ELISA procedure as previously described.[2] Anticanalicular autoantibodies reactive with canaliculi within parietal cells were detected using an immunohistochemical method.[2] To investigate cross reactivity, 60 µl of each serum diluted 1/10 with PBS-Tween were incubated overnight at room temperature with 60 µl of three different *H pylori* lysates (200 µg sonicate/ml): *H pylori* strain W 14, which expresses Lewis X only; *H pylori* strain 700, which expresses high levels of both Lewis X and Lewis Y; and a cocktail of 10 different clinical isolates of *H pylori* positive for both Lewis antigens. Absorption studies using an *Escherishia coli* lysate (200 µg sonicate/ml) or 60 µl PBS-Tween were performed as controls. After incubation, 20 µl of the absorption mixture were further diluted with PBS-Tween to a final serum concentration of 1/500 and were transferred to the ELISA to determine anti-*H pylori* immunoreactivity. The remaining 100 µl of the mixtures were further diluted with 400 µl RPMI and were transferred to immunohistochemical analysis as described above.

Figure 1 Effective absorption of sera to H pylori. Immunoreactivity against H pylori is only reduced after incubation with H pylori but not with E coli or PBS.
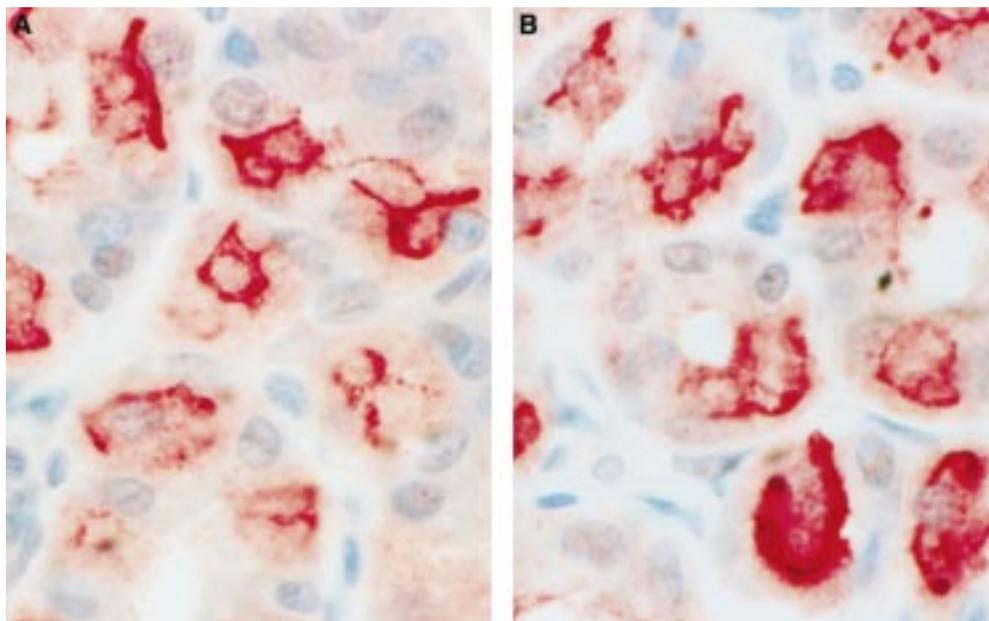
Figure 2   Immunoreactivity of serum S16 against canaliculi of parietal cells in the human gastric mucosa after absorption to PBS (A) and to H pylori (B) (original magnification ×100).

Results

All patients had chronic active *H pylori* gastritis and positive *H pylori* serology. Antigastric autoantibodies against canaliculi within human parietal cells were detectable in all patients. After absorption of the sera to *H pylori,* the reactivity of all sera to *H pylori* was profoundly reduced and fell below the cut off value. In contrast, absorption to *E coli* or PBS-Tween did not alter the immunoreactivity to *H pylori* (fig 1). Antigastric autoreactivity of all sera against human gastric epithelial cells did not change after absorption, in particular the samples effectively absorbed to each of the three different *H pylori* preparations did not convert to negative but remained positive at the same intensity as the controls. A representative immunohistochemical result is shown in fig 2.

Discussion

Negrini *et al* reported that antigastric autoreactivity was abolished after preabsorption of the sera to *H pylori,* indicating that these autoantibodies are cross reacting.[1 3] This was supported by the molecular mimicry between *H pylori* and the host at the level of carbohydrate Lewis blood group antigens.[6] However, as the titres of anti-Lewis X or anti-Lewis Y antibodies in the sera of *H pylori* infected humans are low (Appelmelk, unpublished data), and antigastric autoreactivity persisted after preabsorption to *H pylori* in this study, the idea of molecular mimicry as the pathogenic mechanism leading to antigastric autoimmunity in chronic *H pylori* gastritis must be challenged. The different observations made by Negrini *et al* and by us might be caused by differences in the *H pylori* strains used in the absorption assays, or in the different populations investigated. However, the results presented in our study strongly indicate that autoimmune pathways other than molecular mimicry and novel epitopes are responsible for the antigastric autoreactivity in chronic *H pylori* gastritis.

It is possible that the inflammation itself—that is, the acquisition of a mucosa associated lymphoid tissue (MALT) in the gastric mucosa, and the de novo expression of both major histocompatibility class II antigens and costimulatory molecules on gastric epithelial cells[7 8] meet the requirements for antigastric autoimmune reactions.[9]

Unravelling the mechanisms leading to antigastric autoimmunity in *H pylori* infection provides new and important information about the pathogenesis of *H pylori* gastritis and its complications.

1 Negrini R, Lisato L, Zanella I, *et al.* Helicobacter pylori infection induces antibodies cross-reacting with human gastric mucosa. *Gastroenterology* 1991;**101**:437–45.
2 Faller G, Steininger H, Eck M, *et al.* Antigastric autoantibodies in Helicobacter pylori gastritis: prevalence, in-situ binding sites and clues for clinical relevance. *Virchows Arch* 1996;**427**:483–6.
3 Negrini R, Savio A, Poiesi C, *et al.* Antigenic mimicry between Helicobacter pylori and gastric mucosa in the pathogenesis of body gastritis. *Gastroenterology* 1996;**111**: 655–65.
4 Faller G, Steininger H, Kränzlein J, *et al.* Antigastric autoantibodies in Helicobacter pylori infection: Implications of histological and clinical parameters of gastritis. *Gut* 1997;**47**:619–23.
5 Aspinall GO, Monteiro MA, Pang H, *et al.* Lipopolysaccharide of the Helicobacter pylori type strain NCTC 11637 (ATCC 43504): structure of the O antigen chain and core oligosaccharide regions. *Biochemistry* 1996;**35**:2489–97.
6 Appelmelk BJ, Simoons-Smit I, Negrini R, *et al.* Potential role of molecular mimicry between Helicobacter pylori lipopolysaccharide and host lewis blood group antigens in autoimmunity. *Infect Immun* 1996;**64**;2031–40.
7 Engstrand L, Scheynius A, Pahlson C, *et al.* Association of Campylobacter pylori with induced expression of class II transplantation antigens on gastric epithelial cells. *Infect Immun* 1989;**57**:827–32.
8 Ye G, Barrera C, Fan X, *et al.* Expression of B7–1 and B7–2 costimulatory molecules by human gastric epithelial cells. *J Clin Invest* 1997;**99**:1628–36.
9 Kirchner T, Steininger H, Faller G. Immunopathology of Helicobacter pylori gastritis. *Digestion* 1997;**58**(suppl 1): 14–16.

A pseudo-cryptococcal artefact derived from leucocytes in wet India ink mounts of centrifuged cerebrospinal fluid

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Abstract
Wet India ink mounts of cerebrospinal fluid (CSF) are useful in the laboratory diagnosis of cryptococcal meningitis. Pseudo-cryptococcal artefacts in such mounts have been attributed to leucocytes in CSF but their mode of formation has not been explained. This report describes the reproduction of such an artefact in cryptococcus free CSF-leucocyte mixtures that had been subjected to high speed centrifugation. The viscosity of DNA that could provide a morphological pseudo-capsule, and the yellow-green fluorescence of the pseudo-capsular material on staining with acridine-orange, suggest that lymphocytic nuclear DNA, which possibly leaked out after damage to the lymphocyte membrane by centrifugation, was responsible for this artefact.
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Cryptococcosis is increasing in importance as an opportunistic infection because of the increasing use of therapeutic immunosuppression, increasing incidence of HIV infection, and increase in numbers of susceptible aging people. Conventional laboratory methods for the diagnosis of cryptococcal meningitis with cerebrospinal fluid (CSF) include microscopy (wet India ink negative stain), capsular antigen detection (capsular antigen sensitised latex agglutination test), and culture of *Cryptococcus neoformans*.

Wet India ink mounts of CSF have sometimes shown elements that resemble capsulated cells of cryptococcus (fig 1), although culture on conventional media for fungi and tests for capsular antigen gave negative results. These elements were considered artefacts that probably originated from leucocytes in the CSF. As far as we are aware only one comment, "Lysed lymphocytes in an India ink preparation of a CSF sample have been mistaken for *Cryptococcus neoformans*," exists in the literature[1] on this artefact; no information, however, was given on the mode of its formation.

This report describes the reproduction of this artefact in cryptococcus free CSF mixtures with normal human leucocytes that had been centrifuged.

Materials and methods

CEREBROSPINAL FLUID
Samples of surplus CSF after routine laboratory tests, which were negative (by microscopy and culture on blood agar at 37°C) for aerobic bacteria and for cryptococcus (wet India ink microscopy, tests for capsular antigen by the latex agglutination test (Latex-Crypto antigen detection system; Immuno-Mycologics, Inc, Norman, Oklahoma, USA), and culture on Sabouraud dextrose agar at 30°C), were used in all the tests. Different batches of such CSF were used in replicate tests.

LEUCOCYTES
Buffy coat cells in freshly donated blood (or within seven days of collection) were treated with Tris-ammonium chloride buffer (pH 7.2) to lyse contaminating red cells, washed, and resuspended in physiological saline for addition to CSF.

CSF containing leucocytes (approximately 100 leucocytes/ml) were subjected to a variety of treatments; a fresh mixture was used for each set of tests:
(1) incubation at 25–29°C and at 37°C in air, at 37°C in 5% CO_2, and at 4°C in air, for seven days, with daily examination
(2) evaporation and concentration by leaving the container with its cap loosened, on the bench at room temperature (25–29°C) for seven days, with daily examination
(3) sonication (Labsonic 2000 (Braun, Germany), 8 mm probe) in an ice bath for variable periods from 30 seconds to four minutes
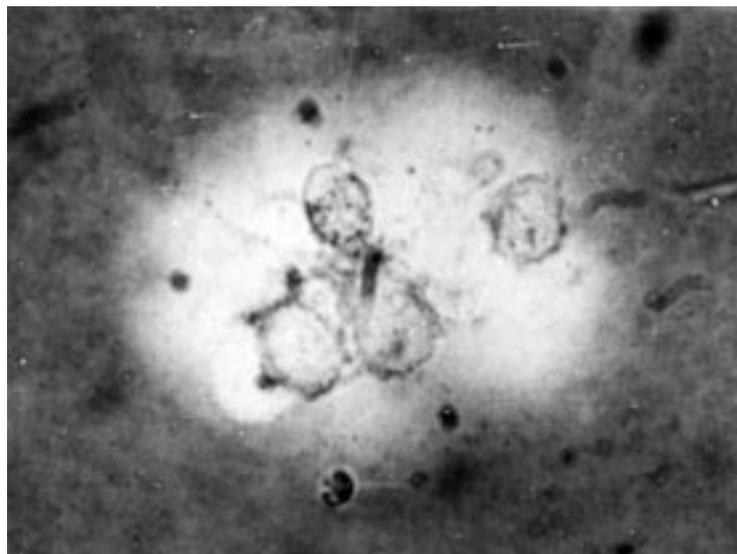

Figure 1   Pseudo-cryptococcal artefact in a clinical specimen of centrifuged CSF from a patient suspected of having meningitis (wet India ink mount).
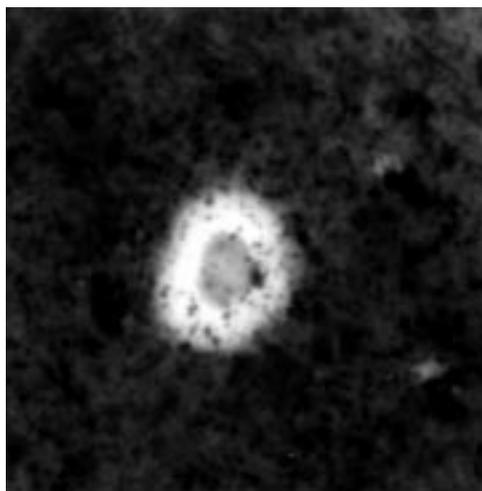
Figure 2 Pseudo-cryptococcal artefact in CSF-leucocyte mixture after centrifugation at 28 000 ×g for 15 minutes at 4°C (wet India-ink mount).

(4) centrifugation at 26°C at 650, 1458, 2590, and 4000 ×g for 15 minutes at each speed, and at 4°C at 6000, 13 500, 24 000, and 54 000 ×g for 15 minutes, at each speed. The deposit was resuspended in a quarter of the original volume of the suspension for the preparation of wet India ink mounts, and smears for staining with acridine orange.

Buffy coat leucocytes, diluted 10-fold in physiological saline, for comparison with CSF-leucocyte mixtures, were centrifuged at 6000 ×g at 4°C for 15 minutes and the deposit was resuspended in a quarter volume of saline for similar tests.

Wet India ink mounts were made on the samples, treated as described above, with an equal volume of commercial India ink (Pelikan, Hannover, Germany) scanned under ×25 and ×40 objectives.

Smears of CSF-leucocyte mixtures and of buffy coat leucocytes in saline that contained the artefacts in India ink mounts were fixed by mild warmth as for bacterial smears, stained with 0.01% aqueous acridine orange, and examined under ultraviolet light with a blue filter, with ×25 and ×40 objectives.

Results

The only treatment of CSF-leucocyte mixtures that resulted in the production of the pseudo-cryptococcal artefacts (fig 2) was centrifugation at 6000 ×g at 4°C. Greater intensities of centrifugation caused disruption of the leucocytes.

In CSF-leucocyte mixtures that produced this artefact some leucocytes appeared distorted or crenated, their nuclei were not clearly demarcated, and it was not possible to distinguish neutrophils from lymphocytes. These artefacts were not of lysed leucocytes, as has been described,[1] because in most preparations the leucocyte cell was visible within the pseudocapsule (fig 2). These abnormal shapes and pseudocapsulation were also seen with some leucocytes in saline, without CSF, that had also been centrifuged at 28 000 ×g. Such

pseudocapsulated leucocytes were absent in preparations of buffy coat leucocytes in saline that had been centrifuged at 365 ×g comparable with the absence of this artefact in CSF-leucocyte mixtures that had also been centrifuged at this lower speed.

On acridine-orange stained smears, these artefacts in CSF-leucocyte mixtures showed a central, yellow-green fluorescence, indicating the presence of the leucocyte's nucleus, with peripheral stippling outside the cell, also yellow-green in colour, suggesting the presence of DNA (whether in pure form or with other nuclear or cytoplasmic constituents) around the cell. Such peripheral stippled fluorescence was not seen on similarly stained smears around buffy coat leucocytes, without CSF, which had been centrifuged at 365 ×g, and which had no pseudocapsular artefacts, as mentioned earlier.

Discussion

These artefacts could prompt a diagnosis of cryptococcosis despite a negative result with the latex agglutination test for cryptococcal capsular antigen and negative culture, when either the numbers of putative cryptococcal cells are small or when the yeast is non-viable because of prior antifungal treatment.

Although it was claimed that lysed lymphocytes could produce such artefacts,[1] the leucocytes in the present study were visible within the pseudocapsule. This suggests that the viscous DNA from the nucleus of the leucocyte had leaked out from the cell following the centrifugation to produce the pseudocapsule. The production of the leucocyte derived artefact is probably attributable to damage of the leucocyte's cell membrane by centrifugation, leading to egress of intracellular DNA, which was viscous enough to simulate a capsule. If this did occur, it has a parallel in the use of centrifugation to enhance infection of cells in the shell vial culture technique with chlamydia and cytomegalovirus. The effect of centrifugation at relatively low speeds on infection of cells by these microorganisms is not caused by mere sedimentation of these microorganisms with the cells, but probably results from alterations in the permeability of the cell membrane allowing entry of these microorganisms into the cells. In the case of our leucocytes, the centrifugation and consequent damage to their cell membranes could have caused the egress of intra-leucocytic DNA. The intensity of centrifugation (6000 ×g) that produced this artefact in our experimental CSF-leucocyte mixtures is, however, not usually used on clinical specimens of CSF in the examination for bacteria and yeasts. The appearance of these artefacts in conventionally processed clinical specimens (with relatively low speeds of centrifugation of 1458 ×g or less), as in fig 1, and not in the experimental CSF-leucocyte mixtures centrifuged at similar speeds, is difficult to explain. It could, however, be due to the effect of unidentified factors in the CSF, such as chemical (drugs) or biological constituents, similar to the effect of polyethylene glycol in

the hybridoma technique, combined with the low intensities of centrifugation.

Giemsa stained smears of a patient's CSF that had this artefact showed only lymphocytes without neutrophils, indicating that it was the lymphocyte from which this artefact arose.

Our results suggest that if sufficient numbers of cryptococci are present on a Gram stained smear, centrifugation should be avoided before microscopy with the India ink technique. If centrifugation is to be used, caution is warranted in interpreting such artefacts as cryptococcus, especially when the capsular antigen detection test and culture give negative results.

1 Merz WG, Roberts GD. Detection and recovery of fungi from clinical specimens. In: Balows A, ed. *Manual of clinical microbiology*. Washington DC: American Society for Microbiology, 1991:550.

Measurement of haemoglobin using single drops of skin puncture blood: is precision acceptable?

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Abstract
The study aimed to investigate local concerns about clinically important discrepancies between repeat HemoCue haemoglobin measurements from single drops of blood. Two biomedical scientists and two health visitors each obtained a series of paired haemoglobin values by fingerprick sampling from healthy volunteers. Seven of 20 paired values obtained by health visitors and three of 20 obtained by scientists from the first drop of blood forming at the puncture site differed by $\geq$ 10 g/l; 11 of 20 paired values obtained by health visitors and one of 20 by the scientists from the fourth drop of blood differed by $\geq$ 10 g/l. After collecting and mixing a number of drops in EDTA tubes before analysis, seven of 40 paired values differed by > 5 g/l, and none by > 10 g/l. Pooling drops of blood before analysis improves precision of HemoCue haemoglobin measurement and allows users to achieve results comparable to those obtained by experienced laboratory staff. Measurement of haemoglobin from single drops of skin puncture blood should be discontinued.
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Keywords: haemoglobin measurement; skin puncture blood sampling

The HemoCue portable haemoglobinometer (HemoCue Ltd, Sheffield, UK) has been available commercially for some years and has been found to be reliable and to give precise and accurate results.[1 2] Nevertheless, concerns exist among local general practitioners about clinically important differences sometimes found between repeat HemoCue measurements made on single drops of skin puncture (capillary) blood. This study was performed to investigate the problem.

Subjects and methods
Two biomedical scientists and two health visitors took blood from four groups of 10 healthy adult volunteers. The health visitors had experience of using HemoCue in their daily work, and the biomedical scientists had considerable experience of skin puncture blood collection. All had received training by staff of HemoCue Ltd in the proper use of the instrument. Three samples were collected from one standard lancet puncture to the middle finger of each hand, producing three paired haemoglobin measurements from each volunteer. Blood from the first and fourth drops forming at the site was collected into HemoCue cuvettes, that from the second and third drops were wiped away with a sterile swab. About 20 drops were then taken into a miniaturised EDTA tube (Teklab Ltd, Co Durham, UK). After mixing, the haemoglobin of this sample was measured using both HemoCue and a Bayer H1 automated blood counter (Bayer plc, Newbury, Berks, UK). Venous blood from both arms of a further 10 volunteers was collected by one biomedical scientist and haemoglobin measured using both instruments. Coefficients of variation obtained by the health visitors and scientists on each sample type were determined, as was the mean and the 95% confidence intervals of the differences between duplicate measurements. Differences between paired values were assessed using the Wilcoxon signed rank test.

Results
Figure 1 shows the differences between paired values obtained with HemoCue; the results are summarised in table 1. Seven of 20 paired values obtained by health visitors and three of 20 samples obtained by the biomedical scientists from the first drop of blood differed by $\geq$ 10 g/l. Eleven of 20 paired values obtained by health visitors and one of 20 obtained by the biomedical scientists from the fourth drop
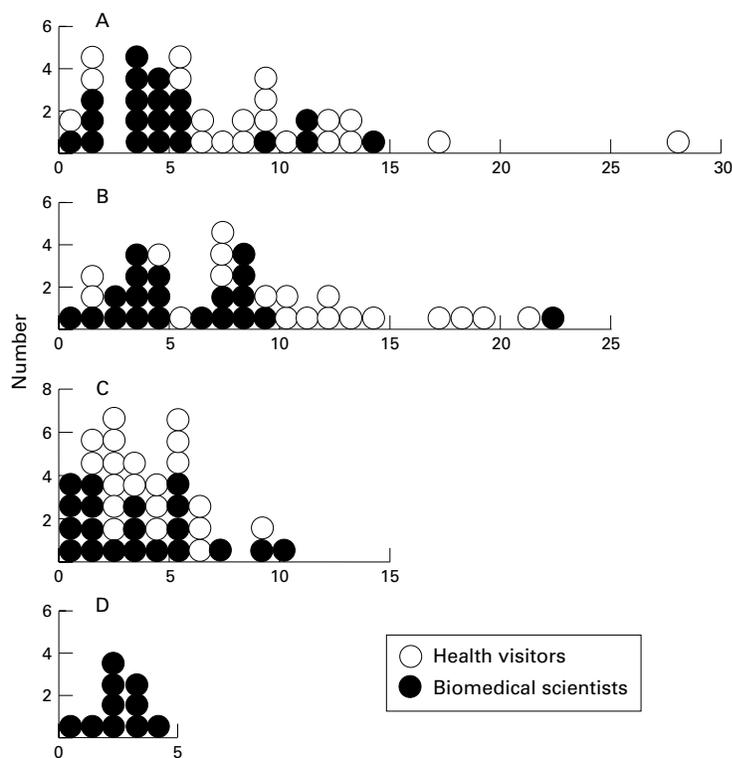
Figure 1   Difference between paired HemoCue haemoglobin measurements (g/l). (A) First drop of skin puncture blood. (B) Fourth drop of skin puncture blood. (C) Pooled drops of skin puncture blood. (D) Venous blood.

Discussion

This brief study confirms a high incidence of disturbingly wide variation between duplicate HemoCue measurements made on single drops of blood collected directly from the skin puncture site. Most of the discrepant results were obtained by the health visitors, despite their being among the most experienced local users of HemoCue. These occurred despite our use of a homogeneous group of healthy and willing volunteers to rule out, as far as possible, the problems sometimes associated with skin puncture sampling in a patient population, such as poor peripheral circulation or lack of cooperation. The health visitors tended to work slowly, sometimes allowing a large drop of blood to form before filling the cuvette. On several occasions there was almost instantaneous sedimentation of red cells in such drops, and sampling from this non-homogeneous source probably caused some discrepancies.

A mean difference between duplicate skin puncture haemoglobin measurements of 5 g/l, with 10% of paired values differing by $\geq$ 10g/l, has previously been reported.[3] These results are almost identical to those obtained with single drops of blood by the biomedical scientists in the present study. Although details of sample collection were not given in the earlier report, the similarity leads us to suggest that these figures are the best precision obtainable with this type of sample. In a patient population this degree of precision may be unobtainable.

Mills and Meadows[4] showed improved precision of haemoglobin measurement using HemoCue is possible by pooling and mixing drops of skin puncture blood before analysis. Despite this report, the manufacturer continues to recommend the use of a single drop of blood for analysis and, based on its own studies, suggests the fourth drop forming at the puncture site provides the most representative sample. Our results do not support this contention, but confirm the previous report and extend it to show the precision of haemoglobin measurement in pooled drops of skin puncture blood approaches that obtainable with venous blood under ideal conditions—that is, where samples are collected by an experienced phlebotomist from healthy volunteers with good veins. Furthermore, this improved precision was obtained by both biomedical scientists and health visitors, despite the latter

of blood differed by $\geq$ 10 g/l. No paired values from the pooled blood differed by > 10 g/l; four obtained by health visitors and three by biomedical scientists differed by > 5 g/l. Differences between paired values obtained by the biomedical scientists were significantly lower than those of the health visitors for both the first and fourth drops of blood (p < 0.01). Differences between paired values obtained by the health visitors on pooled drops using HemoCue were significantly lower than those they obtained on both the first and fourth drops (p < 0.001). The precision of HemoCue compared well with that of the automated analyser on both the larger volume skin puncture and venous samples. Haemoglobin values obtained ranged from 108–172 g/l: mean 137.5 g/l, HemoCue, first drop; 137.8 g/l, HemoCue, fourth drop; 141.7 g/l HemoCue, pooled drops; and 137.2 g/l, Bayer H1, pooled drops.

Table 1   Coefficients of variation, mean differences between duplicate haemoglobin measurements, and 95% confidence intervals of the differences obtained by both groups of workers on each type of blood sample

Sample	Instrument	Biomedical scientists				Health visitors			
		No of paired values	CV (%)	Mean difference between duplicate samples (g/l)	95% CI	No of paired values	CV (%)	Mean difference between duplicate samples (g/l)	95% CI
Skin puncture blood									
First drop	HemoCue	20	2.9	4.7	3.0 to 6.4	20	5.4	8.9	6.1 to 11.7
Fourth drop	HemoCue	20	3.6	5.7	3.6 to 7.8	20	6.3	10.5	7.8 to 13.2
Pooled drops	HemoCue	20	2.1	3.2	1.9 to 4.5	20	2.1	3.3	2.1 to 4.5
Pooled drops	Bayer H1	20	1.2	1.9	1.3 to 2.5	20	1.8	2.7	1.7 to 3.7
Venous blood	HemoCue	10	1.2	2.2	1.5 to 2.9				
Venous blood	Bayer H1	10	1.5	2.5	1.5 to 3.5				

never having used this method of collection before.

This study has confirmed the precision of haemoglobin measurement using HemoCue, and shown that skin puncture blood samples can be simply and reliably used with this instrument with little training, provided a number of drops are anticoagulated and mixed before analysis. In contrast, analysis of single drops of blood, even by experienced personnel, can give rise to misleading results. This approach to haemoglobin measurement should be discontinued.

1 Neville RG. Evaluation of portable haemoglobinometer in general practice. *BMJ* 1987;**294**:1263–5.
2 Sharp S, Lewis SM, Williams SK. Evaluation of the HemoCue B-haemoglobin photometer. *Medical Devices Agency evaluation report MDA/95/21* London: HMSO, 1995.
3 Reeves JD, Vinchinsky E, Addiego J Jr, *et al.* Iron deficiency in health and disease. In: Barnes LA, ed. *Advances in pediatrics*. Chicago: Year Book Medical Publishers 1983:281–319.
4 Mills AF, Meadows N. Screening for anaemia: evaluation of a haemoglobinometer. *Arch Dis Child* 1989;**64**:1468–71.

Unacceptably high site variability in postmortem blood alcohol analysis

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Abstract
Blood alcohol concentration is a frequently requested test in forensic pathology. The variability of this value was studied by measuring the blood alcohol concentration from six sites in nine subjects at necropsy in whom alcohol was the implicated cause of death. There were small consistent differences in the blood alcohol concentrations between the sites in the nine subjects (p < 0.04). Calculation of the mean blood:vitreous humour alcohol concentration ratio (B:V ratio) showed that vitreous humour alcohol concentration most closely reflected the concentration at the femoral vein (B:V ratio = 0.94, r = 0.98), which is considered the optimal site for blood alcohol measurement. The correlation of left heart blood with femoral blood was lower compared with the other sites. There is a potential for an unacceptably large variation in the postmortem measurement of blood alcohol within each subject.
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Keywords: postmortem examination; blood alcohol concentration; vitreous humour

The measurement of postmortem blood alcohol concentration is one of the most commonly requested tests in forensic pathology. The importance of this measurement is clear as it is used as evidence in court to determine the part played by alcohol in both criminal and civil trials. Great care must be taken in the sampling and storage of postmortem blood used to measure alcohol concentration to avoid artefactual changes from—for example, putrefaction.[1] Despite such considerations, variability in blood alcohol concentration has been found between different sites of the same cadaveric specimen.[2 3] One explanation for this is postmortem diffusion of alcohol from the gastric lumen into neighbouring blood vessels and viscera.[3 4] The principal aim of this study was to determine the degree of this variability by measuring blood alcohol concentrations from five separate vascular sites in nine cadaveric subjects. We also studied whether this variability had any bearing on the use of vitreous humour alcohol measurement to estimate blood alcohol concentration.

Methods

Blood samples were collected from nine consecutive subjects in whom alcohol was implicated in the cause of death. All subjects had been involved in road traffic accidents with minimal delay in refrigeration of the cadaver (within four hours of death) so that putrefaction should have been negligible. Samples from axillary and femoral sites were collected by percutaneous external puncture before the start of the necropsy; cardiac blood was collected by opening the pericardial sac and aspirating from the right and left ventricles; and the internal jugular vein was dissected and sampled directly. The samples were obtained before dissection of the abdominal contents or disturbance of the viscera. Blood (10 ml) was collected from each of these five sites from each subject. Collection of vitreous humour involved adducting the eye and inserting the needle through the lateral sclera; 2 ml of vitreous humour was collected from each subject. All specimens were collected by one person using separate 20 ml syringes and 18 gauge needles for each site. Vitreous humour was not sampled from one subject (subject 3) as the eyes had been damaged.

The samples were immediately transferred into 2% fluoridated tubes and frozen at −18°C until further analysis. All samples were analysed by headspace gas chromatography as a single batch by the same operator. All assays were performed in duplicate and mean values

Table 1   Postmortem alcohol concentrations (mg/100 ml) from nine subjects

Sampling site	Subject									B:V ratio*	r†
	1	2	3	4	5	6	7	8	9		
Femoral vein	134	256	360	265	26	236	213	102	244	0.94	0.98
Axillary vein	125	253	350	241	16	227	208	80	263	0.85	0.96
Left ventricle	101	354	363	229	298	239	166	118	324	2.33	0.26
Right ventricle	103	274	340	219	34	203	182	58	268	0.87	0.91
Internal jugular vein	87	246	344	230	19	218	205	57	225	0.77	0.97
Vitreous humour	138	294	–	294	25	239	249	121	230	–	–

*Mean blood:vitreous humour alcohol concentration ratio; †Pearson correlation coefficient for blood and vitreous humour alcohol concentrations.

(mg/100 ml) used for comparison and statistical analysis of variance by the ANOVA test. The mean blood:vitreous alcohol concentration ratio (B:V ratio) and the Pearson correlation coefficient for blood and vitreous humour alcohol concentrations were calculated for each of the five vascular sampling sites. The legal and ethical aspects of this research have met the requirements of the Human Tissue Act of Victoria, Australia (1982).

Results

All subjects had at least one blood alcohol concentration value over 80 mg/100 ml (table 1). There were small consistent differences in the blood alcohol concentrations between the sites in the nine subjects ($F = 2.62$, $p < 0.04$). Within these data there is an aberrant value of 298 mg/100 ml from the left heart blood of subject 5. If the ANOVA is repeated excluding this value, which is statistically preferable,[5] the differences between the sites remain ($F = 3.37$, $p < 0.01$). Comparison of the B:V ratios and the correlation coefficients showed that of the five vascular sampling sites, vitreous humour alcohol concentration most closely reflected that of femoral vein blood (table 1). It was also apparent that the correlation coefficient of left heart blood alcohol concentration with vitreous humour alcohol concentration was low ($r = 0.26$) compared with other sites. If the aberrant value from subject 5 is excluded, the correlation coefficient improves to 0.75, which, while higher, is well below that of the other vascular sites.

Discussion

These results confirm that there is a potential for error in the analysis of postmortem blood alcohol concentration. In one of the subjects (subject 5), the left ventricular alcohol concentration was more than 10 times that of the femoral vein. While it is accepted that this value may be erroneously high, the sample analyses were performed in duplicate suggesting that this result cannot be ignored entirely. In addition, the alcohol concentration in the left ventricular blood of subject 2 was much higher than in samples collected from the other five sites. It has been demonstrated in cadaveric models that alcohol can diffuse from the gastric lumen into the left ventricle.[4] While this study did not seek to demonstrate this mechanism, the magnitude of the error illustrates how easily indiscriminate postmortem blood sampling can alter the significance given to alcohol as a factor contributing to the circumstances and cause of death. In particular, the results suggest

that higher values may occur in left heart blood and that this site does not correlate well with the other vascular sites studied; therefore it should not be trusted to provide consistently accurate values.

There has been much debate as to whether blood alcohol concentrations can be reliably extrapolated from vitreous humour alcohol concentrations.[6 7] This issue may need further assessment as several previous studies relied on comparison with blood sampled from the heart or aorta.[8 9] Because of its distance from the stomach and the pulmonary venous system, the femoral vein has been proposed as the optimal site for the measurement of postmortem blood alcohol concentration.[4] Interestingly, within our small study population, vitreous humour alcohol concentration most closely reflected that of femoral vein blood. The ratio of blood to vitreous humour alcohol concentration has been shown to vary between the absorption and elimination phases of in vivo alcohol metabolism.[7] Larger studies are required before vitreous humour alcohol measurement can be promoted as an alternative to femoral vein alcohol measurement. A larger study population, knowledge of the timing between alcohol ingestion, death, and postmortem sampling would allow investigation of the relation between femoral blood and vitreous humour alcohol concentration.

Standard sampling protocols for the measurement of blood alcohol at necropsy are followed in the specialist forensic unit.[10] Adherence to these may be less stringent in general pathology practice. There should be an enforced, standard protocol for the measurement of postmortem blood alcohol concentration. The protocol should outline the optimal method and site of collection, storage, and analysis. Furthermore, whenever postmortem blood alcohol measurement is done, there should be accurate documentation of the circumstances of death, cause of death, the times of alcohol ingestion and death, and the state of the body noting, in particular, the extent of decomposition. Minimising measurement error and taking into account these variables should help improve on the current situation where erroneous conclusions are potentially being drawn because of nonstandardised postmortem blood alcohol measurement.

1 O'Neal CL, Poklis A. Postmortem production of ethanol and factors that influence interpretation: a critical review. *Am J Forensic Med Pathol* 1996;**17**:8–20.
2 Briglia EJ, Bidanset JH, Dal Cortivo LA. The distribution of ethanol in postmortem blood specimens. *J Forensic Sci* 1992;**37**:991–8.

3 Marraccini JV, Carroll T, Grant S, *et al*. Differences between multisite postmortem ethanol concentrations as related to agonal events. *J Forensic Sci* 1990;**35**:1360–3.

4 Pounder DJ, Smith DRW. Postmortem diffusion of alcohol from the stomach. *Am J Forensic Med Pathol* 1995;**16**:89–96.

5 Healy MJR, Whitehead TP. Outlying values in the National Quality Control Scheme. *Ann Clin Biochem* 1980,**17**:78–81.

6 Caplan YH, Levine B. Vitreous humour in the evaluation of postmortem blood ethanol concentrations. *J Anal Toxicol* 1990;**14**:305–7.

7 Tzee CC, Lo DST. Relationship between postmortem blood and vitreous humor ethanol levels. *Am J Forensic Med Pathol* 1993;**14**:303–8.

8 Coe JI, Sherman RE. Comparative study of postmortem vitreous humor and blood alcohol. *J Forensic Sci* 1970;**15**:185–90.

9 Winer CL, Esposito FM. Comparative study of ethanol levels in blood versus bone marrow, vitreous humor, bile and urine. *Forensic Sci Int* 1981;**17**:27–36.

10 PA Toseland. Samples and sampling. Analytical techniques. In: Moffat AC, ed. *Clarke's isolation and identification of drugs, pharmaceuticals, body fluids and post-mortem material*, 2nd ed. London: The Pharmaceutical Press, 1986:111–17.