Effect of pH changes in cerebrospinal fluid specimens on bacterial survival and antigen test results

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Abstract

Aims—To determine the effect of pH changes occurring in cerebrospinal fluid (CSF) after sampling on the viability of meningitis causing bacteria, and on the performance of agglutination assays used for the rapid detection of bacterial antigens.

Methods—The pH of CSF collected via lumbar puncture was measured by various methods, and the effects of the following different incubation conditions on subsequent changes were determined: air at 4°C; air at room temperature (22°C); air at 37°C; and air with 5% CO₂ at 37°C. The growth/survival in pooled CSF of 15 bacterial isolates collected from 74 patients with meningitis was assessed in these incubation conditions over 24 hours. The effects of pH changes in the CSF on the sensitivity of two latex agglutination and one co-agglutination kits for detecting *Haemophilus influenzae*, *Neisseria meningitidis* groups B and C, and *Streptococcus pneumoniae* were determined.

Results—The measured pH of CSF was highly affected by the method used and particularly the time delay between patient sampling and assay. Measured pH values at the time of sampling (mean 7.5) increased rapidly within 60 seconds by about one unit. CSF pH continued to increase during incubation in all tested conditions (up to approximately pH 10), with the exception of in air with 5% CO₂ at 37°C where pH changes were reversible and near physiological values were attained. Bacterial survival for all species tested was poorest in CSF incubated in air at 37°C and best following exposure to air with 5% CO₂ at 37°C. Agglutination in rapid antigen detection kits with CSF incubated in air as opposed to air with 5% CO₂ generally took longer to occur and in some instances was less prominent. In one case a false negative result was obtained with CSF seeded with *N meningitidis* group B incubated in the former but not the latter conditions.

Conclusions—CSF pH increases after patient sampling are minimised and/or mostly reversed by incubation in an atmosphere containing 5% CO₂. CSF samples should ideally be placed in such an atmosphere as soon as possible after collection, and left there until laboratory processing occurs, to reduce the detri-

mental effects of pH stress on bacterial survival. pH increases may also reduce the likelihood of obtaining a positive result in rapid antigen detection assays.

(J Clin Pathol 1996;49:249-253)

Keywords: bacteria, pH, cerebrospinal fluid, diagnosis of meningitis.

In vivo body fluids are buffered to a physiological pH by shifts in the equilibrium reaction between carbonic acid and carbon dioxide (CO₂) and water. Removal of body fluids from their buffered environment, not surprisingly, leads to pH changes. For example, the pH of human serum and used peritoneal dialysis fluid increases after collection to approximately 8.5-9.0 because of diffusion of CO₂ out of the specimens. This is associated with the precipitation of proteins and some metallic cations.^{1,2} Previous studies with such body fluids have shown that bacterial growth/survival is adversely affected by pH increases.¹ However, these pH changes and consequent biochemical disturbances are preventable (and partially reversible) if body fluids are placed in an atmosphere of air with 5% CO₂. Conventionally, microbiology laboratories use an atmosphere of air with 5% CO₂ to culture many bacteria, primarily because of the improved growth obtained in the presence of such physiological CO₂ tensions.

Earlier studies indicate that the pH of cerebrospinal fluid (CSF) increases almost immediately after removal from the body, but the magnitude of such changes over a longer period and the consequences for bacterial survival have not been evaluated.³⁻⁵ We speculated that pH changes occurring in CSF, collected either by lumbar puncture or via ventricular drains, may reduce the viability of meningitis causing bacteria before laboratory processing. This would lessen the likelihood of obtaining a positive CSF culture result, which is vital for accurate determination of the aetiology, treatment, prophylaxis, and epidemiology of bacterial meningitis. We were also interested to ascertain whether alterations in the pH of CSF might affect the performance of agglutination assays which are sometimes used for the rapid detection of bacterial antigens.

Methods

COLLECTION, POOLING AND STORAGE OF CSF

Two sources of CSF were used to create a pool for bacterial survival experiments. The residual fluid after processing of CSF specimens sent

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Accepted for publication
21 November 1995

to the diagnostic laboratory, over a three month period, was stored at -20°C on the same day of receipt. Specimens which were culture positive, xanthochromic, or contained significantly raised numbers of white or red blood cells (>100 cells/litre) were omitted from the pool. CSF was also collected at the bedside during lumbar puncture, in patients with known or suspected multiple sclerosis undergoing diagnostic taps and in patients with benign intracranial hypertension undergoing therapeutic taps. These specimens were frozen at -20°C within one hour of collection. CSF was also collected via ventricular drains from six patients, although all of these specimens were either xanthochromic or contained antibiotics (see below) and therefore were not included in the CSF pool.

All CSF specimens were checked for bacterial contamination and antimicrobial activity and positives were omitted from the pool. CSF specimens from patients known to be receiving antibiotics at the time of sampling were not included in the pool. Bacterial growth was detected by streaking $100\ \mu\text{l}$ CSF on a blood agar plate and then incubating in air with 5% CO_2 for 24 hours. Antimicrobial activity was detected by placing $20\ \mu\text{l}$ CSF into wells in two blood agar plates which had been seeded with *Staphylococcus aureus* NCTC 6571 or a clinical isolate of *Streptococcus pneumoniae*. Plates were checked for zones of inhibition after incubation in air with 5% CO_2 for 24 hours. The lower limit of sensitivity of this assay for cefotaxime was approximately $0.3\ \text{mg/l}$.

Further manipulations of CSF were kept to a minimum, and in particular freeze-thaw cycles were avoided wherever possible. Frozen CSF was thawed in an incubator containing air with 5% CO_2 at 37°C . The final CSF pool (approximately $250\ \text{ml}$) was assimilated from 74 patients.

MEASUREMENT OF CSF pH

Three techniques were used to measure CSF pH at the time of routine lumbar puncture (in patients without meningitis). Firstly, CSF was collected in a polystyrene specimen container, and the probe of a hand-held pH meter (pH Boy, Camlab, Cambridge, UK) was then immersed into the fluid within 60 seconds of sampling (four patients). This pH meter was used throughout the study because of the small sample volume (about $20\ \mu\text{l}$) required to obtain a reading, and was calibrated daily. Secondly, CSF was allowed to drip from the lumbar puncture cannula directly on to the pH probe (13 patients). Finally, a $2\ \text{ml}$ syringe was connected to the lumbar puncture cannula and approximately $1\ \text{ml}$ CSF was gently aspirated; this CSF was expressed directly on to the pH probe within 30 seconds of sampling (four patients). The pH of CSF obtained (usually over the course of several days) via a ventricular drain was measured by probe immersion at the time of collection (six patients).

EFFECT OF INCUBATION CONDITIONS ON CSF pH

Four non-meningitic CSF specimens obtained via lumbar puncture from different patients

were immediately transported to the laboratory. Each specimen was split into four $1\ \text{ml}$ volumes which were placed in $5\ \text{ml}$ plastic bijoux. The bijoux were placed in one of four incubation conditions: air at 4°C ; air at room temperature (22°C); air at 37°C ; and air with 5% CO_2 at 37°C . pH measurements were taken by immersion of the probe, taking care to clean the probe with 70% ethanol and distilled water between readings, at the following times: 0, 10, 20, 30, 40, 50, 60, 80, 100, and 120 minutes, and four and 24 hours. At the end of these experiments, $100\ \mu\text{l}$ of each CSF aliquot was plated onto blood agar for overnight incubation to exclude bacterial contamination.

BIOCHEMISTRY OF POOLED CSF

Pooled CSF ($20\ \mu\text{l}$) was thawed in air with 5% CO_2 at 37°C , and then divided into four equal volumes in centrifuge-type universal containers. The containers were placed in the four different incubation conditions for 24 hours. Following centrifugation at $1000\times g$ for five minutes, supernatant fluids were carefully decanted from any precipitate, and then immediately analysed for electrolyte and protein concentrations using an autoanalyser.

EFFECT OF INCUBATION CONDITIONS ON BACTERIAL SURVIVAL IN CSF

Fifteen clinical isolates from the CSF of patients with meningitis were examined in growth/survival experiments; these comprised three *Neisseria meningitidis* (two group C and one group B), three *S. pneumoniae*, three *Haemophilus influenzae*, three *Staphylococcus epidermidis*, two *Pseudomonas aeruginosa*, and one *Escherichia coli*. The strains had been stored on beads in glycerol broth at -70°C , and were subcultured on blood or chocolate blood agar the day before use. Inocula were prepared from these plates, after overnight incubation in air with 5% CO_2 at 37°C , and in each case four $1\ \text{ml}$ aliquots of pooled CSF (in $3\ \text{ml}$ plastic bijoux with loose fitting caps) were seeded with equal numbers of a bacterial strain to a final concentration of approximately 10^4 – 10^5 cfu/ml. The bijoux were placed in the four different incubation conditions, and $10\ \mu\text{l}$ aliquots were removed at zero, six and 24 hours for enumeration of viable bacteria by serial 10-fold dilutions. The experiments were repeated but with the following sampling times: 0, 30, 60, 90, and 120 minutes, and 24 hours. Aliquots of sterile pooled CSF were used throughout as controls, and to monitor pH changes. A similar series of bacterial survival experiments was also carried out, using antibiotic-free xanthochromic CSF obtained over a five day period via a bilateral ventricular drain from one patient. This CSF was centrifuged at $1000\times g$ for five minutes to remove intact erythrocytes.

EFFECT OF CSF INCUBATION CONDITIONS ON BACTERIAL ANTIGEN DETECTION

The effects CSF pH on the performance of two latex agglutination kits (Directigen Meningitis

Table 1 Effect of incubation conditions (24 hours) on CSF pH and biochemical measurements

Biochemical measurements	Incubation conditions			
	Air at 4°C	Air at 22°C	Air at 37°C	Air with 5% CO ₂ at 37°C
pH	9.4	9.5	10.2	7.5
Ca (mmol/l)	1.02	1.03	1.02	1.04
Mg (mmol/l)	1.05	1.03	1.08	1.16
PO ₄ (mmol/l)	0.44	0.44	0.41	0.44
Protein (g/l)	0.7	0.5	0.5	1.0

Combo Test, Becton Dickinson, Oxford; and Slidex Meningite kit, bioMérieux, Basingstoke, Hampshire, UK) and one coagglutination kit (Phadebact CSF test, Launch Diagnostics, Longfield, Kent, UK) used for the rapid detection of bacterial antigens were investigated. All kits were in date and appropriate results were seen with the respective controls. Aliquots (0.75 ml) of CSF obtained from one patient via a ventricular drain which had antimicrobial activity, but which was free of bacterial contamination, were seeded with one of the following meningitis causing strains to give a starting concentration of 10⁸ cfu/ml: *H influenzae*, *N meningitidis* group B, *N meningitidis* group C, and *S pneumoniae*. Each seeded CSF sample was heated at 100°C in a water bath for five minutes, although it is stated that optimum sensitivity for the detection of *N meningitidis* group B in the Directigen Test is obtained with unheated CSF. For each CSF sample, two series of 10-fold dilutions (in heated CSF) were made in separate 96 well, round bottomed microtitre trays. One series of trays was placed in air at 37°C and the other in air with 5% CO₂ at 37°C for 90 minutes. Control plates containing sterile heated CSF were used to monitor changes in CSF pH. Following incubation, appropriate assays for bacterial antigens in the CSF dilutions were carried out immediately, according to the manufacturers' instructions, recording the time taken for agglutination to occur.

Results

EFFECT OF INCUBATION CONDITIONS ON CSF pH
The observed pHs of CSF samples obtained at routine lumbar puncture in patients without meningitis were highly method dependent. Immersion of the probe in CSF (n=4), direct sampling on to the pH probe (n=13) and syringe aspiration (n=4) gave mean (range) pH values of 8.4 (7.8–9.1), 7.7 (7.3–8.1) and 7.5 (7.4–7.6), respectively. The mean pH of CSF obtained via ventricular drains (n=6) was 9.0.

Figure 1 shows the typical effects of incubation conditions on the pH of one CSF sample. Notably, rapid increases in CSF pH occurred within a few seconds of exposure to an atmosphere without a physiological level of CO₂; higher incubation temperatures exacerbated this phenomenon. High CSF pHs were almost completely reversible by exposure to physiological levels of CO₂. Similar effects of incubation conditions on pH were observed with xanthochromic CSF (data not shown).

Figure 1 Effect of incubation conditions on CSF pH.

BIOCHEMISTRY OF POOLED CSF

Table 1 shows the biochemical parameters of CSF following overnight incubation in the four incubation conditions, and subsequent centrifugation to remove any precipitate. The major difference was a 50% reduction in the concentration of solubilised proteins in CSF which had been incubated in air at either 22°C or 37°C compared with incubation in air with 5% CO₂ at 37°C.

EFFECT OF INCUBATION CONDITIONS ON BACTERIAL SURVIVAL IN CSF

The results for the two series of experiments, involving different sampling times, were very similar, and for convenience only one set is presented. Bacterial survival/growth was in almost all cases better following incubation in air with 5% CO₂ at 37°C than in any of the other conditions (figs 2A–2F). Overall, the poorest survival was observed following incubation in air at 37°C. Survival was generally improved in xanthochromic CSF compared with pooled CSF. *E coli* and *P aeruginosa* actually grew in all the incubation conditions, with the exception of air at 4°C when bacterial numbers remained static up to 24 hours (data not shown). All three *S epidermidis* strains examined grew in air with 5% CO₂ at 37°C, but the bacteria were

Table 2 Effect of incubation conditions on time taken (seconds) for agglutination to occur

Bacterial strain	Incubation conditions	
	Air	Air with 5% CO ₂
<i>H influenzae</i>		
Directigen	70	60
Phadebact	35	25
Slidex	120(±)*	90
<i>N meningitidis</i> group B		
Directigen	—†	90
Phadebact	60	30
Slidex	40	45
<i>S pneumoniae</i>		
Directigen	40	50
Phadebact	60(±)*	60
Slidex	120(±)*	120
<i>N meningitidis</i> group C		
Directigen	45	40
Phadebact	60(±)*	60(±)*
Slidex	30	20

* Very fine agglutination(±); † no agglutination observed after 10 minutes.

Figure 2 Survival of one clinical isolate each of *H influenzae* (A, B), *N meningitidis* (C, D) and *S pneumoniae* (E, F) in pooled (A, C, E) and xanthochromic (B, D, F) CSF held in four different incubation conditions: air at 4°C; air at room temperature (22°C); air at 37°C; and air with 5% CO₂ at 37°C.

killed by incubation in air at 37°C, with intermediate results obtained in the remaining incubation conditions (data not shown).

EFFECT OF CSF INCUBATION CONDITIONS ON BACTERIAL ANTIGEN DETECTION

The antigen detection kits were uniformly of low sensitivity and no positive results were observed with bacterial concentrations <10⁷ cfu/ml. In general, agglutination appeared sooner and was of better quality with CSF samples that had been incubated in air with 5% CO₂ compared with air alone (table 2). It was notable that no agglutination was observed using the Directigen kit to detect *N meningitidis* group B in CSF which had been incubated in air, in contrast to bacteria in CSF exposed to

air with 5% CO₂. Similar results were obtained in repeat experiments (data not shown). The pH of control CSF samples was 9.7 and 7.7 following incubation in air and air with 5% CO₂, respectively. In separate experiments it was observed that although increases occurred following boiling of CSF, a difference between the pH of samples that had been preincubated in air or air with 5% CO₂ at 37°C was still maintained; pre- and post-boiling CSF pHs increased from 7.3 to 8.7 or 9.7, respectively, after preincubation in these conditions.

Discussion

The optimal pHs for *N meningitidis* and *S pneumoniae*, the main bacterial pathogens causing meningitis, are 7.4–7.6 and 7.8, re-

spectively, although growth will occur over a wider pH range (for example, pH 6.5–8.3 for pneumococci).^{6,7} CSF is poorly buffered and so a rapid increase in pH occurs once it is removed from an environment containing CO₂. Higher incubation temperatures exacerbate this phenomenon, presumably because of increased diffusion of CO₂ from the CSF sample. Not surprisingly, the increased pH ultimately leads to the death of many of the fastidious bacteria that cause the great majority of episodes of meningitis.

Our findings emphasise the importance of the rapid transportation of CSF from the patient to the laboratory. The incubation conditions chosen for study represent those to which CSF specimens may routinely be exposed before laboratory processing.⁸ The pH changes which occur in CSF after removal from the patient can be minimised and/or mostly reversed by placing specimens in an atmosphere containing 5% CO₂. Therefore, CSF samples should ideally be placed in such an atmosphere as soon as possible after collection from the patient, and left there until laboratory processing occurs. We were largely unable to detect bacterial death during the first two hours of sub-optimal incubation conditions, but these observations need to be interpreted with some caution. The presence of antibiotic and phagocytic white cells in CSF is likely to reduce the numbers of viable bacteria, and every reasonable effort should be taken to limit further stress, such as that caused by rises in pH. Also, we have not assessed whether pH stress may render bacteria relatively non-culturable. The presence of blood in CSF led to increased bacterial survival/growth. This may be due to the nutrient qualities of blood or its buffering capacity. Although CSF specimens from patients with suspected meningitis are generally transported rapidly to the laboratory, some samples are not always processed directly upon receipt—for example, those from cases of suspected subarachnoid haemorrhage or from individuals with CSF drainage devices. In such cases unexpected infections may not be identified if bacteria are exposed to prolonged pH stress.

It is possible that factors other than the increased pH per se could be bactericidal. For example, it has been shown that one and two hours after collection of CSF, the numbers of intact neutrophils in specimens held at room temperature decrease by 32% and 50%, respectively.⁹ Numbers of lymphocytes and monocytes did not decrease significantly during the same period. This may be caused by the movement of water and solutes from the

extracellular to intracellular compartments, caused by the hypotonicity of CSF, pH changes, reduced concentrations of membrane stabilising proteins and/or lipids, or a combination of these factors. Neutrophil lysis would result in the release of enzymes and other degradative compounds which would be expected to have an adverse effect on bacterial survival. Precipitation of proteins and other factors from CSF may also be detrimental to bacterial survival.

Changes in CSF pH may adversely affect the complexing of antigen with antibody, possibly due to conformational changes in surface epitopes. It could be argued that the false negative result with CSF exposed to air for *N meningitidis* group B is caused by heating the sample (which is not recommended by the manufacturer). However, the corresponding sample gave a positive result, despite being heated, following incubation in air with 5% CO₂. Also, it is somewhat impractical to split the often small available volume of CSF in the diagnostic laboratory, not heating one aliquot before testing for *N meningitidis* group B, and heating another prior to processing for the remaining bacterial antigens. We, like others,¹⁰ are extremely doubtful as to the usefulness of rapid antigen testing for the diagnosis of bacterial meningitis, primarily because of the low sensitivity of the latex agglutination kits. Our results show that pH changes in CSF may further reduce the likelihood of obtaining a positive result in these assays.

We thank Becton Dickinson, bioMérieux, and Launch Diagnostics for providing the antigen detection kits.

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