LETTERS TO THE EDITOR

Rapid identification of *Chlamydia psittaci* and TWAR (C pneumoniae) in sputum samples using an amplified enzyme immunoassay

Recent publications on the importance of the TWAR agent^{1,2} and our own experience of *Chlamydia psittaci* infections have led us to conduct a prospective study of routine sputum samples from patients with respiratory infections using a commercial enzyme linked immunosorbent assay (EIA) for detecting chlamydia antigen.³

One hundred and six sputa were received from 100 patients with acute or persisting chest infections and with no known underlying or predisposing condition. Sera were requested whenever possible and were obtained from 30 patients. Three sputa from patients not fulfilling the trial criteria but yielding a pure heavy growth of *Staphylococcus aureus* were included to confirm the non-reactivity of this organism in this assay.

All sputa were cultured routinely to establish their bacteriological profile. Sputum specimens for *Chlamydia* detection were tested by the IDEIA Chlamydia test (Novo BioLabs Ltd) and sera from patients, where available, were tested by routine complement fixation test and by a modification of a previously described enzyme immunoassay (EIA) for *Chlamydia* group-specific IgM.⁴ This modification required a 1 in 400 dilution of serum (100 µl) and a first incubation step of 90 minutes at 37°C. After washing, incubation with antigen took place at 37°C for one hour and conjugate was added for a further one hour at 37°C. Remaining aspects of the test were as previously described. Specimens positive by the EIA antigen test were also tested by direct immunofluorescence for direct visualisation of elementary bodies with characteristic morphology, using a species specific major outer membrane protein (MOMP) reactive monoclonal antibody (SYVA Microtrak) and a genus specific lipopolysaccharide (LPS) monoclonal (Imagen, Novo BioLabs Ltd).

Preparation for antigen EIA detection required mucolysis of sputum in 2% buffered n-acetyl cysteine before washing and centrifugation. The deposit was resuspended in the transport medium provided by the manufacturers. Samples positive by EIA were centrifuged as before, resuspended in PBS, and smears were examined by direct immunofluorescence for both genus and species specific fluorescence.

Results

No clinically important pathogens were detected in 79 of the 106 sputa examined. A variety of other organisms were detected including *Haemophilus influenzae* (n = 7), *Streptococcus pneumoniae* (n = 1), *S aureus* (n = 6), *Pseudomonas* sp (n = 4), *Aspergillus* sp (n = 1) and yeasts (n = 1).

Seven sputa from five patients were positive by IDEIA Chlamydia test and also by Imagen *Chlamydia* direct immunofluorescence but negative by Syva Microtrak, indicating that the organism was *C psittaci* or *C pneumoniae*. In all cases the chlamydia IgM test was positive and in four cases the complement fixation titre was raised, indicating recent chlamydial infection.

One specimen was positive by all three chlamydia tests, indicating *C trachomatis* infection. Although the complement fixation titre was less than 16, the specific IgM was positive and species antibody was raised.

One specimen from a patient (case 6) with serological evidence of chlamydial infection was found to be weakly positive by Imagen but negative by EIA and Syva Microtrak. This specimen was salivary and probably inadequate.

All other sputum samples were negative by IDEIA Chlamydia test including the three specimens with heavy *S aureus* growth. All sera from patients other than those mentioned above had undetectable specific IgM and complement fixation titres of < 32.

The laboratory findings of the seven patients with evidence of chlamydial infection are shown in the table.

Our initial findings indicate that testing of routine bacteriological sputa by IDEIA Chlamydia test may be useful for rapid detection of chlamydial respiratory infections. This is substantiated by the direct immunofluorescence studies on the sputa and also by serology.

Although a wide variety of bacteria were encountered in this study, there were no problems with cross reactions. Four of the seven chlamydial infections described here

would not have been diagnosed by routine procedures.

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Diagnosis of toxoplasmosis using DNA probes

During the past 15 years it has become increasingly evident that patients being immunosuppressed for malignant disease or organ transplantation may develop generalised toxoplasmosis which affects the central nervous system and may result in death.¹ Recent findings have shown that patients with AIDS constitute a further group prone to severe disseminated toxoplasmosis²; in such patients toxoplasmosis constitutes a life-threatening opportunistic infection. The current methods for the diagnosis of toxoplasmosis have given dismayingly poor results in such patients and, therefore, there is a need for more sensitive procedures for the early diagnosis of *Toxoplasma gondii* in body fluids or biopsy tissues.

A good alternative to the serological tests may be the detection of the pathogen's nucleic acids in clinical specimens. Over the past few years the application of recombinant DNA procedures has led to the development of a number of DNA probes for potential use in the diagnosis of pathogens such as *Neisseria gonorrhoeae*, *Plasmodium falciparum*, cytomegalovirus and enterotoxigenic *Escherichia coli*.³ Recent advances in recombinant DNA technology, and in particular the development of the polymerase chain reaction (PCR),⁴ allow certain pathogens to be detected which may be present in very small numbers in a given sample.

We wanted to develop a DNA based test for the diagnosis of *T gondii*. We used the PCR (30 cycles) and two oligonucleotide probes (20-mers) based on the published sequence for the toxoplasma P30 gene⁵ to amplify toxoplasma DNA. These reactions were performed with purified toxoplasma DNA (0-100 ng) in the presence or absence of 5 µg human DNA (this corresponds to about 1.5 × 10⁶ leucocytes). On electrophoresis of the reactions on 0.8% (w/v) agarose gels and subsequent staining of the gel with ethidium bromide, amplified product (915 base pairs long) could be seen in those reactions contain-

Laboratory findings of seven respiratory chlamydial infections

Case No	Clinical details	Date of sputum sample	Antigen detection		Date of serum	Serology	
			EIA (od)	Direct immuno-fluorescence (LPS)		Titre	IgM
1	Cough for 4 weeks, dust contact	14.3.89	+0.958	+	12/4	128	+
2	Chronic cough, radiographic changes	25.5.89	+ <2	+	1/6	>128	+
3	Chest infection acquired a budgie recently	12.5.89	+0.178	+	8/5	>128	+
4	Haemoptysis	20.6.89	+ >2	+			
5	Cough, no bird contact	2.6.89	+0.468	+	1/6	<16*	+
6	Chest infection, radiographic changes	5.7.89	+1.309	+	11/7	16	+
7	Chest infection, parrot died	11.7.89	+0.279	+	2/8	16	+
		28.7.89	-	+	21/7	>128	+
		salivary			7/8	>128	+
		19.8.89	+0.263	+	21/8	128	+

(a) Southern hybridisation of amplified *T gondii* DNA, and (b) electrophoretic analysis of the second PCR. In both cases, the top and bottom rows of samples are in the absence and in the presence of human DNA, respectively. The amount of toxoplasma DNA added to each reaction is shown in picograms.

ing as little as 10 pg toxoplasma DNA. In the presence of human DNA this was obscured by a smear of human DNA (presumably this was the result of the 30 cycles of heating and cooling of the DNA together with some non-specific amplification). When the DNA was transferred from the gel on to a nylon membrane (Southern blot) and then hybridised to a radiolabelled probe (corresponding to the amplified DNA), however, the desired amplification product could be seen in reactions containing as little as 0.5 pg toxoplasma DNA, which corresponds to about two organisms. Furthermore, as shown in fig 1a, this was detectable both in the presence and in the absence of any human DNA. Essentially similar results were obtained with dot blots or slot blots instead of Southern blots and with non-radioactive probes.

Because the PCR/hybridisation assay requires at least two days we developed an assay requiring no hybridisation. After the PCR, aliquots of the 915 base pair DNA fragment are amplified further using two different oligonucleotides within this fragment. The results indicate that after the second PCR a 520 base pair fragment of DNA is clearly visible on agarose gels both in the presence and in the absence of human DNA (fig 1b). Using this procedure a result can be obtained in about seven to eight hours.

Although the work described here is still at an early stage, we have used it to examine

some clinical samples, including a brain biopsy specimen from a patient with AIDS (unpublished observations). The results were extremely promising and suggest that diagnosis of toxoplasmosis using the PCR and DNA probes may soon become a routine alternative to serological tests.

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Evaluation of 3-fucosyl N-acetylglucosamine antibody staining in histological assessment of CIN

Expression of 3-fucosyl N-acetylglucosamine (CD15) antigen by non-keratinising squamous epithelia gives an indication of cell maturity and might therefore prove valuable in improving consistency of cervical intra-epithelial neoplasia (CIN) grading.¹⁻³ MC2 is a monoclonal antibody raised against human granulocytes⁴ which recognises the CD15 antigen. In biopsy specimens of normal cervix the squamous epithelium shows a broad zone of staining in suprabasal cells: the depth of this MC2 stained zone progressively reduces with increasing severity of CIN grade.¹

Thirty six cervical biopsy specimens were examined by 11 consultant histopathologists: for each case there was a section stained with haematoxylin and eosin and a serial section stained for CD15 by an indirect avidin-biotin immunoperoxidase method. The pathologists initially graded the lesions using the haematoxylin and eosin section; thereafter the sections were regraded, but also now taking into account the CD15 stained section in an attempt to improve the consistency of diagnosis. Details of the proforma used for recording the results and the statistical analysis by κ statistics have been described previously.⁵ The κ statistics are a measure of

overall agreement which do not require any assumption concerning a "gold standard" correct diagnosis. The statistical method includes a correction for the amount of agreement which could be expected by chance alone.

Table 1 presents the κ statistics for agreement on the presence and degree of CIN. When the haematoxylin and eosin stained slides were studied alone overall agreement was fair ($\kappa=0.40$), but this was largely due to good agreement on the CIN 3 and normal categories: the results for the CIN 1 and 2 categories showed poor agreement. Addition of the slide stained with CD15 resulted in slight reduction in the overall level of agreement ($\kappa=0.34$). There was no improvement in the assessment of CIN grades 1 and 2 and there was a slight reduction in the level of agreement on those biopsy specimens which had been reported as normal. The table also gives the κ statistics for agreement on the presence of viral features in the specimens. The level of agreement is poor with the haematoxylin and eosin stained section alone ($\kappa=0.18$) and shows no improvement when the CD15 stained slide is also used in the assessment ($\kappa=0.14$).

In a previous study we showed good agreement among histopathologists in the diagnosis of CIN 3 but not for the lesser grades of CIN.⁵ In view of the problems associated with interpreting cervical biopsy specimens using only a standard haematoxylin and eosin stained section and the reported suggestion that use of a CD15 stained section might improve discrimination, we had expected that this study would result in improved consistency of reporting. Although there seemed to be an association between a decrease in CD15 staining and increasing severity of CIN, the consistency of grading, unfortunately, did not improve significantly. The reasons for this are uncertain but may reflect a subconscious unwillingness on the part of the pathologist to be swayed diagnostically by a CD15 stained section, preferring instead to rely more on the well accustomed use of the standard haematoxylin and eosin stained section. Alternatively, the localisation of CD15 may not have been sufficiently precise, or the antigen may have been dispersed too irregularly, to permit critical distinction of the lower grades of CIN, the area of greatest diagnostic difficulty.

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Table 1 *Kappa statistics*

Agreement on CIN categories:		Normal	CIN1	CIN2	CIN3(+)	Overall
Haematoxylin and eosin alone	K	0.48	0.08	0.20	0.64	0.40
Haematoxylin and eosin and MC2	K	0.33	0.11	0.13	0.66	0.34
Comparison	K	0.79	0.51	0.55	0.78	0.69
Agreement on viral features:		None	Outwith CIN	Within CIN	Both	Overall
Haematoxylin and eosin alone	K	0.19	0.22	0.09	0.20	0.18
Haematoxylin and eosin and MC2	K	0.17	0.16	0.08	0.14	0.14
Comparison	K	0.79	0.72	0.86	0.77	0.78