It is interesting to note that in the present study LH-RH treatment resulted in cytomorphological changes in only one cell type whereas in mammals it affects the cytomorphology of two cell types namely LH and FSH gonadotrops[3]. Therefore, it is suggested that the pars distalis of *R. cyanophlyctis* contains one type of gonadotrop represented by the B2 cell type and the hormone(s) secreted by B2 cells regulate the spermatogenetic and steroidogenic activity of the testis in *R. cyanophlyctis*.

1 The free gift of LH-RH from NIH is gratefully acknowledged. Authors are grateful to CSIR, New Delhi, for the award of a SRF to the first author and to Karnatak University, Dharwad for providing laboratory facilities.
2 Schally, A. V., Science *202* (1978) 18.
3 Gregerson, K. A., and Campbell, G. T., Biol. Reprod. *27* (1982) 169.
4 Licht, P., and Papkoff, H., Endocrinology *94* (1974) 1587.
5 Daniels, E. and Licht, P., Gen. comp. Endocr. *42* (1980) 455.
6 McCreery, B. R., and Licht, P., Biol. Reprod. *29* (1983) 129.
7 Kasinathan, S., Guna Singh, A., and Basu, S. L., Acta morph. Acad. Sci. hung. *26* (1978) 187.
8 Oordt, P. G. W. J. van, Gen. comp. Endocr. *40* (1980) 308.
9 Iwasawa, H., and Kera, Y., Gen. comp. Endocr. *46* (1982) 236.
10 Mazzi, V., Vellano, C., Colucci, D., and Merlo, M., Gen. comp. Endocr. *24* (1974) 1.
11 Andreoletti, G. E., Mazzi, V., Vellano, C., and Sacerdote, M., Monitore zool. ital. *14* (1980) 255.
12 Yajurvedi, H. N., and Hooli, M. A., Proc. Indian natn. Sci. Acad., part B, *49* (1983) 620.
13 Saidapur, S. K., and Nadkarni, V. B., Indian J. exp. Biol. *10* (1972) 425.
14 Oordt, P. G. W. J. van, Regulation of spermatogenetic cycle in the common frog, *(Rana temporaria)*. Van Der Weil, Arnhem 1956.
15 Oordt, P. G. W. J. van, in: Physiology of Amphibia, vol. 2, p. 53. Ed. B. Lofts. Academic Press, New York 1974.
16 Doerr-Schott, J., Gen. comp. Endocr. *28* (1976) 487.
17 Kime, D. E., Vinson, G. P., Patricia, W., and Kilpatric, R., in: General comparative and clinical endocrinology of adreanal cortex, vol. 3, p. 183. Eds G. I. Chester Jones and I. W. Handerson. Academic Press, New York 1981.
18 Lofts, B., in: Physiology of Amphibia, vol. 2, p. 107. Ed. B. Lofts. Academic Press, New York 1974.

0014-4754/85/081058-03$1.50 + 0.20/0
© Birkhäuser Verlag Basel, 1985

Clomiphene citrate can mimic the augmentative (positive) but not the depressing (negative) effect of estradiol on the LHRH-stimulated release of LH and FSH by the pituitary gland of the long-term ovariectomized rat

G. A. Schuiling, N. Pols-Valkhof and T. R. Koiter

Division of Experimental Endocrinology, Department of Obstetrics and Gynaecology, University of Groningen, 59 Oostersingel, 9713 EZ Groningen (The Netherlands), 16 April 1984

Summary. In the long-term ovariectomized rat, both estradiol benzoate (EB) and clomiphene citrate enhance the release of LH induced by luteinizing hormone-releasing hormone (LHRH). EB also enhances the release of FSH. In rats pretreated with LHRH, EB strongly depresses the LHRH-induced LH/FSH release, but clomiphene enhances this release, regardless of the presence of EB.
Key words. Ovariectomized rat; LHRH; estradiol benzoate; clomiphene citrate; LH release.

Clomiphene citrate is a nonsteroidal agent which is frequently and successfully used for induction of ovulation in anovulatory women[3,19]. Despite its widespread use, the drug's mode of action has not been fully clarified, e.g. data obtained with pituitary cell cultures indicate the drug to have both estrogen-agonistic and estrogen-antagonistic properties[9-11,20,28]. It is, however, generally believed that clomiphene acts as a competitive inhibitor of estradiol at hypothalamic and/or pituitary receptor sites[1,12,14].

When estradiol benzoate (EB) is administered to long-term ovariectomized (OVX) rats, the elevated, pulsatile secretion of LH of such animals decreases almost instantaneously [3]. This acute effect of estrogen is not due to suppression of the hypothalamic LHRH pulses, but to decreased pituitary LHRH-responsiveness[24]. However, about 9 h after administration of EB the pituitary LHRH-responsiveness begins to increase markedly, a development which coincides with the EB-induced suppression of the hypothalamic LHRH secretion[22,23]. This might suggest that suppression of the hypothalamic LHRH secretion is involved in the estrogen-induced sensitization of the pituitary gland. In this study we tested this hypothesis and re-investigated the mode of action of clomiphene on the pituitary gland in 2-week OVX rats. One series of rats was pretreated with exogenous LHRH, delivered by s.c. implanted Alzet osmotic minipumps, in order to prevent lowering of LHRH exposure of the pituitary gland due to EB-induced suppression of the hypothalamic LHRH secretion. The LHRH-releasing minipumps were therefore implanted before administration of EB. Another series of OVX rats was not pretreated with LHRH.

Materials and methods. Wistar rats were ovariectomized at the age of 3 months and used for experiments 2 weeks later. Ovariectomy was performed to eliminate the influence of ovarian hormones. The general arrangement of the experiments was as follows: some of the rats received LHRH for 6 days at the rate of 250 ng/h (released by Alzet® osmotic minipumps, model 2001, s.c. implanted at 09.00 h on day 1); other rats received a 'sham-pump', i.e. a piece of silastic with the dimensions of a minipump. In all rats responses of LH and FSH were induced on day 6 by continuous infusion (through an intra-jugular cannula) of LHRH at the rate of 1 µg/h for 21 h (the infusions started at 12.00 h). The high dose of LHRH was chosen for methodological reasons: after the described LHRH pretreatment significant LH/FSH responses could only be induced in the rats still bearing the minipumps with a strong stimulus.

Both in the case of the LHRH pretreatment and of the sham-procedure four different pretreatments preceded the LHRH infusions. I: clomiphene (100 µg by s.c. injection) was given 75, 27 and 0 h before the LHRH infusion. II: estradiol benzoate (3 µg by s.c. injection) was given 75 and 27 h before the infusion. III: treatments I and II were combined. IV: control rats received solvent.

Blood samples were taken via an intra-carotid cannula (cannulation was performed at least 2 h before LHRH infusion). Operations were carried out under appropriate ether anesthesia. Plasma LH and FSH concentrations were measured by double antibody radioimmunoassay with NIAMDDK rat LH- and FSH-RP-1 as reference preparations. LH/FSH responses were judged

Analysis of data of figures 1 and 2. Plasma LH and FSH as maximal height (ng LH/FSH-RP-1 per ml plasma) and quantity of LH/FSH secreted from t = 0 to t = 21 h (area units; see 'Materials and methods' and Koiter et al.[15] (means ± SEM) as caused by 21 h of infusion of LHRH at the rate of 1 µg/h

Experimental group		Maximal height LH	Maximal height FSH	Quantity secreted (21 h) LH	Quantity secreted (21 h) FSH
Not pretreated with LHRH	Solvent (n = 8)	5271 ± 536[a]	3092 ± 195[a]	228.7 ± 15.9[a]	195.7 ± 11.1[a]
	EB (n = 7)	14282 ± 1167[b]	5516 ± 285[b]	291.6 ± 16.3[a]	209.4 ± 33.7[a]
	Clomiphene (n = 6)	10235 ± 2514[b]	3530 ± 692[a]	330.3 ± 79.9[a,b]	162.0 ± 36.2[a]
	EB plus clomiphene (n = 6)	13482 ± 1823[b]	4107 ± 600[a]	442.5 ± 44.5[b]	171.8 ± 42.6[a]
		F = 7.88	F = 6.00	F = 4.51	F = 0.48
Pretreated with LHRH	Solvent (n = 6)	2996 ± 104[a,*]	1591 ± 140[a,b]	132.4 ± 7.9[a]	92.3 ± 4.6[a,b]
	EB (n = 6)	860 ± 97[a,*]	776 ± 96[a]	52.1 ± 5.8[b]	60.0 ± 4.6[b]
	Clomiphene (n = 6)	5298 ± 992[b]	2446 ± 508[b]	143.9 ± 18.8[a]	119.0 ± 20.5[a]
	EB plus clomiphene (n = 6)	4944 ± 1081[a,b]	2746 ± 754[b]	141.0 ± 24.2[a]	129.3 ± 24.0[a]
		F = 7.69	F = 3.70	F = 7.40	F = 3.70

EB: estradiol benzoate. Statistical method: see 'Materials and methods'. For each column holds: data without common superscript differ significantly. *p = 0.05.

according to 2 parameters: 1) the maximal height of the responses (MH; ng LH/FSH-RP-1 per ml plasma) and 2) the quantity of LH/FSH secreted during 21 h of LHRH infusion (QS). QS is expressed in arbitarary 'area units', a measure for the area-under-the-curve of the LH/FSH curves (see figs 1 and 2). Under the present experimental conditions QS, or integrated LH/FSH release, is linearly proportional to the amount of LH/FSH secreted during LHRH infusion. See Koiter et al.[15] for method. Data are expressed as mean ±SEM. Statistical comparisons were made by analysis of variance and then by Duncan's multiple comparison test[27]. A difference was considered to be significant when the analysis of variance showed significant heterogeneity for the whole group and the multiple comparison gave a value of $p < 0.05$ for the two groups concerned.

Results and discussion. The present regimen of EB treatment gave plasma estradiol concentrations which at t = 0 h amounted to 22.3 ± 5.1 pg/ml (n = 3), a value well in the pro-estrus range (21.8 ± 0.8 pg/ml; n = 3). In rats not pretreated with LHRH both EB (cf. Ross et al.[19]) and clomiphene (cf. Hsueh et al.[9]) as well as EB plus clomiphene had a positive effect on the secretion of LH induced by LHRH infusion, the effect of clomiphene being slightly weaker than that of EB (fig. 1A; table). Also on the comparable secretion of FSH EB, but not clomiphene, had a positive effect. The combination: EB plus clomiphene tended to be less effective than EB alone, which is probably due to the weaker estrogenic activity of clomiphene (fig. 1B; table).

In animals pretreated with LHRH (250 ng/h for 6 days; fig. 2; table) the LHRH infusions caused LH and FSH responses which were lower in solvent-injected rats than in similar rats not pretreated with LHRH. This phenomenon, already known from previous studies in which LHRH was infused according to the same regimen, is due to the partial depletion of the pituitary LH/FSH stores which is caused by the LHRH pretreatment. These observations thus confirm that though constant exposure of the pituitary gonadotrophs to LHRH may cause 'desensitization' or 'down-regulation' of the mechanisms which underly LH and FSH release [6,23], a pituitary gland, desensitized by a non-maximal LHRH-stimulation, is still able to respond to a higher LHRH concentration: LH/FSH responses to staircase stimulation patterns are additive (cf. Koiter et al.[16]). The same

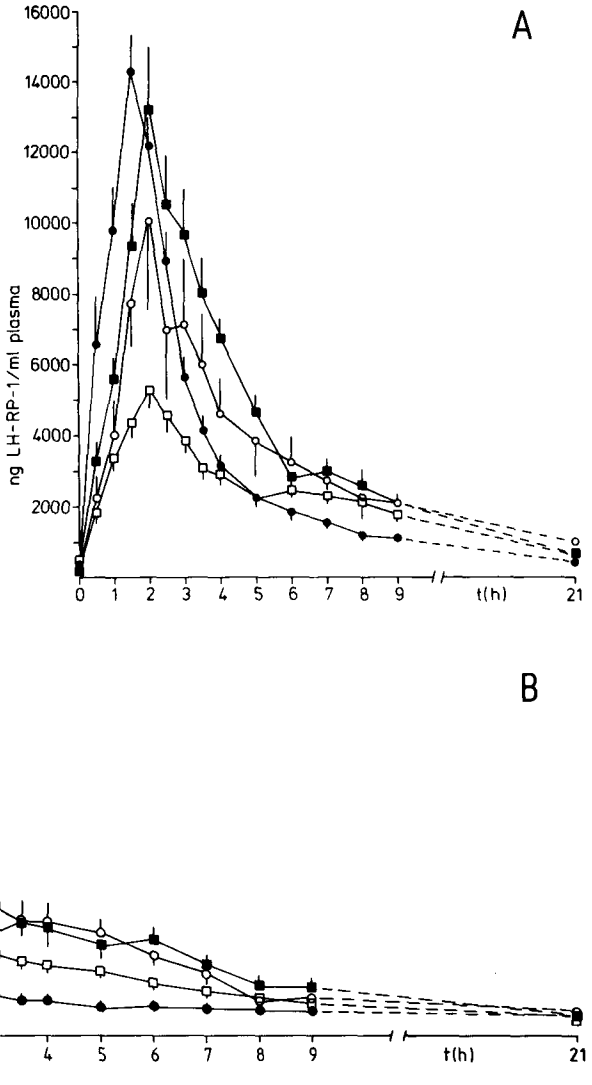

Figure 1. The course of the plasma LH (A) and FSH (B) concentrations (mean ± SEM) during 21 h of constant rate infusion of LHRH (1 µg/h) in OVX rats having been implanted with a silastic 'sham-pump' and injected with: solvent (□—□; n = 8); OB (●—●; n = 7); clomiphene (○—○; n = 6) or with OB plus clomiphene (■—■; n = 6).

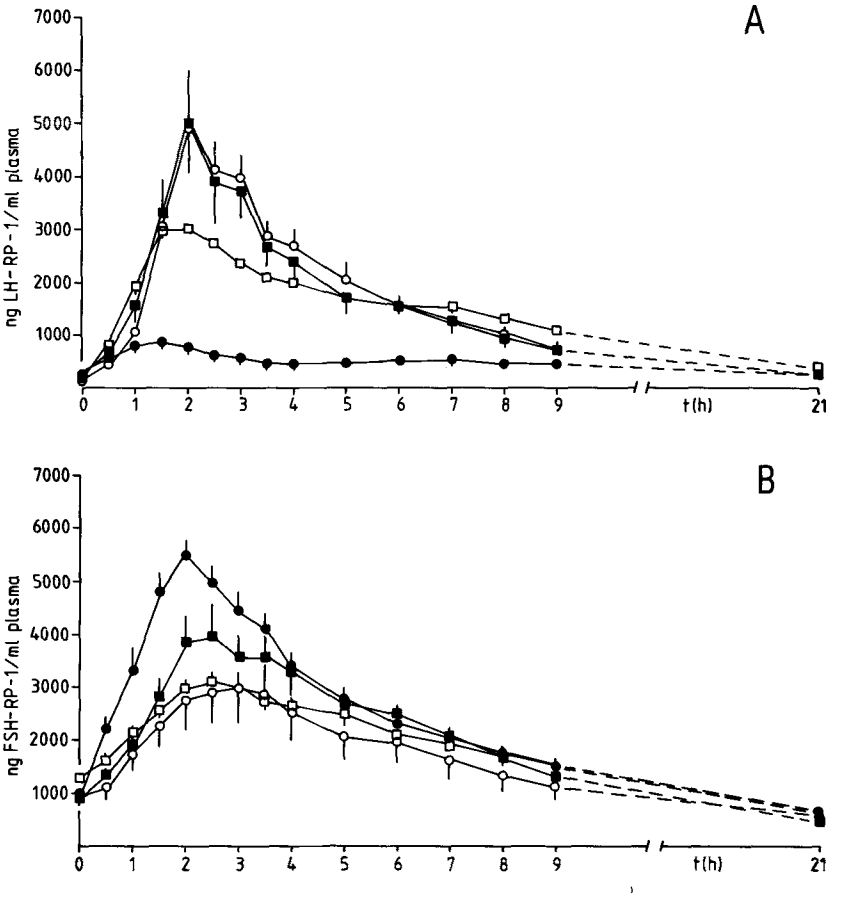

Figure 2. The course of the plasma LH (A) and FSH (B) concentrations (mean ± SEM) during 21 h of constant rate infusion of LHRH (1 µg/h) in OVX rats having been pretreated for 6 days with LHRH, 250 ng/h, by means of s.c. implanted osmotic minipumps, and injected with: solvent (□—□; n = 6); OB (●—●; n = 6); clomiphene (○—○; n = 6) or with OB plus clomiphene (■—■; n = 6).

phenomenon was observed during in vitro studies on the pattern of LH release induced by exposure to LHRH of dispersed pituitary cells[2].

The positive effect of EB, observed in rats not pretreated with LHRH, was not seen in animals bearing LHRH-releasing minipumps for 6 days: in LHRH-pretreated rats injected with EB, the LHRH infusions caused a secretion of LH and FSH which was not enhanced as in the experiments depicted in figure 1, but impaired, suggesting that a pituitary gland, influenced by both LHRH and EB at the same time, is relatively unresponsive to LHRH. Statistically, the negative effect of EB on the LHRH-induced secretion of LH is only significant as far as the quantity of LH secreted is concerned. The mean value of the maximal height of the plasma LH concentration is at the limit of significance ($p = 0.05$). In the case of FSH neither parameter is significant. The reported data, however, support the view that the positive effect of EB cannot develop in the presence of significant concentrations of LHRH (cf. Schuiling and Gnodde[24]).

Although in the experiments depicted in figure 1 clomiphene bahaved like a 'normal' (though weak) estrogen, it did not do so in rats pretreated with LHRH: unlike EB, clomiphene did not exert a negative effect in LHRH-pretreated rats. As in rats not pretreated with LHRH, it enhanced the MH (though not the QS) of the LHRH-stimulated gonadotropin responses. This suggests that at least in the present OVX rats clomiphene, unlike EB, is unable to depress the pituitary LHRH-responsiveness. Also in LHRH-pretreated OVX rats injected with both EB and clomiphene no depression of the LHRH-responsiveness was seen: in these rats clomiphene did not only antagonize the effect of EB (see above), it also increased the LHRH-responsiveness[28]. The reported effects of clomiphene are not easy to understand on the basis of clomiphene being only an estrogen-antagonist. As a weak estrogen it can indeed compete with estradiol, but at the same time clomiphene exerts a positive estrogen-effect on the pituitary LHRH-responsiveness of the OVX rat. It thus seems to lack a property which estradiol (and possibly other estrogens) does possess in this experimental animal, namely the ability to activate the estrogen-sensitive feedback system which is involved in the regulation of the gonadotropin secretion[4].

The present study does not deal with the effect of clomiphene on the hypothalamic secretion of LHRH. However, a stimulatory effect of clomiphene has been demonstrated in vitro using rat hypothalami[17] and there is indirect evidence that in healthy males[7,8,21] and females[29] clomiphene enhances the secretion of LHRH by the hypothalamus while depressing the pituitary LHRH-responsiveness. This depressing effect of clomiphene on the pituitary LHRH-responsiveness of healthy individuals is not necessarily in contradiction to the present results; by competing with endogenous gonadal steroids, notably estrogens, clomiphene may weaken the sensitizing effect of these steroids on the pituitary LHRH-responsiveness. Also, the clomiphene-induced sustained secretion of LHRH by the hypothalamus may cause down-regulation of the LH/FSH secretion mechanisms[23].

Administration of clomiphene may thus lead to stimulation of the hypothalamic LHRH secretion as well as to an increased LHRH-responsiveness of the pituitary gland. These combined effects may start the sequence of events which leads to ovulation in a number of anovulatory women[5,13,18,19].

1 Adashi, E. Y., Hsueh, A. W. J., and Yen, S. S. C., J. Endocr. *87* (1980) 383.
2 Badger, Th. M., Loughlin, J. S., and Nadaff, P. G., Endocrinology *112* (1983) 793.
3 Blake, C. A., Norman, R. L., and Sawyer, C. A., Neuroendocrinology *16* (1974) 22.

4 Bogdanove, E.M., Vitam. Horm. *22* (1964) 205.
5 Czygan, P.G., and Schulz, K.D., Gynaec. Invest. *3* (1972) 126.
6 Catt, K.J., and Dufau, M., A. Rev. Physiol. *39* (1977) 529.
7 Dhont, M.H., DeGezelle, H., and Vandekerckhove, D., Clin. Endocr. *5* (1976) 175.
8 Hashimoto, T., Miyai, K., Matsumoto, K., Izumi, K., and Kumahara, Y., J. clin. Endocr. Metab. *41* (1975) 1110.
9 Hsueh, A.J.W., Erickson, G.F., and Yen, S.S.C., Nature *273* (1978) 57.
10 Hsueh, A.J.W., Erickson, G.F., and Yen, SS.C., Endocrinology *104* (1979) 807.
11 Huang, E.S-R., and Miller, W.L., Endocrinology *112* (1983) 442.
12 Igarashi, M., Ibuki, Y., Kubo, H., Kamoika, J., Yokota, N., Ebara, Y., and Matsu, S., Am.J. Obstet. Gynec. *97* (1968) 120.
13 Jacobson, A., Marshall, J.R., Ross, G.T., and Cargille, C.M., Am. J. Obstet. Gynec. *102* (1968) 284.
14 Kato, J., Kobayashi, T., and Villee, C.A., Endocrinology *82* (1968) 1049.
15 Koiter, T.R., Pols-Valkhof, N., and Schuiling, G.A., Acta endocr. *92* (1979) 28.
16 Koiter, T.R., Pols-Valkhof, N., and Schuiling, G.A., Acta endocr. *96* (1981) 309.
17 Miyake, A., Tasaka, K., Sakumoto, T., Kawamura, Y., Nagahara, Y., and Aono, T., Acta endocr. *103* (1983) 289.
18 Rebar, R.H., Judd, H.L., Yen, S.S.C., Rakoff, J., Vandenberg, G., and Naftolin, F., J. clin. Invest. *57* (1976) 1320.
19 Ross, G.T., Cargille, C.M., Lipsett, M.B., Rayford, P.L., Marshall, J.R., Strott, C.A., and Rodbard, D., Rec. Progr. Horm. Res. *26* (1970) 1.
20 Roy, S., Greenblatt, R.B., and Mahesh, V.B., Acta endocr. *47* (1964) 657.
21 Santen, R.J., and Ruby, E.B., J. clin. Endocr. Metab. *48* (1979) 315.
22 Sarkar, D.K., and Fink, G., J. Endocr. *86* (1980) 511.
23 Schuiling, G.A., and Gnodde, H.P., J. Endocr. *71* (1976) 1.
24 Schuiling, G.A., and Gnodde, H.P., J. Endocr. *72* (1977) 121.
25 Schuiling, G.A., Moes H., and Koiter, T.R., Acta endocr. *106* (1984) 454.
26 Schuiling, G.A., Pols-Valkhof, N., van der Schaaf-Verdonk, G.C.J., and Koiter, T.R., J. Endocr. *103* (1984) 301.
27 Steel, R.G.D., and Torrie, J.H., Principals and procedures of statistics. McGraw-Hill, New York 1960.
28 Vaitukaitis, J.L., Bermudez, J.A., Cargille, C.M., Lipsett, M.B., and Ross, G.T., J. clin. Endocr. Metab. *32* (1971) 503.
29 Wang, C.F., and Yen, S.S.C., J. clin. Invest. *55* (1975) 201.

0014-4754/85/081060-04$1.50 + 0.20/0
© Birkhäuser Verlag Basel, 1985

Comparison of counterimmunoelectrophoresis (CIE), latex agglutination (LA) and staphylococcal coagglutination (COAG) in pneumococcal antigen detection in vitro

M.W. Rytel and M.J. Rytel

Division of Infectious Diseases, Medical College of Wisconsin, Milwaukee County Medical Complex, Milwaukee (Wisconsin 53226, USA), 26 July 1984

Summary. CIE was compared to agglutination assays employing commercial kits (Directigen, Phadebact), as well as our own LA and COAG reagents, in detection of pneumococcal capsular polysaccharide (PCP) antigens in vitro. Directigen provided the most sensitive assay. CIE was of comparable sensitivity except for PCP antigen types 7 and 14.
Key words. Pneumococcal capsular polysaccharides; antigens; antibody; counterimmunoelectrophoresis; latex agglutination; staphylococcal coagglutination.

Etiologic diagnosis in infectious diseases should be established rapidly and accurately to permit optimal therapy. Current research centers on detection of microbial antigens in body fluids. Many different techniques are being evaluated. The purpose of this study was to compare the sensitivity of counterimmunoelectrophoresis (CIE), latex agglutination (LA) and staphylococcal coagglutination (COAG) in detection of pneumococcal capsular polysaccharide (PCP) antigens in vitro. Reagents came from commercial kits or were prepared in our own laboratory.

Latex agglutination (LA). Polystyrene latex particles coated with specific antibodies are reacted with soluble antigens. Agglutination occurs when there is sufficient antigen to cause the sensitized particles to clump together and form a visible aggregate. Two preparations of latex reagents were used in this study, Directigen (Hynson, Westcott & Dunning, Baltimore, Md) and Bacto latex (Difco Laboratories, Detroit, MI) which were sensitized in our laboratory. The sensitization procedure was based on that of Kumar et al.[1] and was briefly as follows: Latex particles, 0.81 μm (undiluted, unwashed) were mixed in equal volume with Omniserum (Statens Seruminstitut, Copenhagen, Denmark), which had been diluted 1:10 in glycine buffered saline (GBS), pH 8.2, 0.1 M. This mixture was then incubated and rotated (Labquake, Labindustries, Berkeley, CA) at 37°C for 3 h, then left to stand at 4°C overnight. This sensitized preparation was diluted 1:2 with 0.1% bovine serum albumin (BSA, 0.1% in GBS) and was then ready to use. Antigen dilutions were prepared from the 14-valent pneumococcal vaccine, Pneumovax™ (Merck, Sharp and Dohme, West Point, PA) containing PCP types 1–4, 6, 8, 9, 12, 14, 19, 23, 25, 51, 56 and individual PCP types 3, 7, 9, 14 (Eli Lilly Laboratories, Indianapolis, IN). The test was run by adding one drop of latex reagent (20 μl) to one drop of antigen, mixing with a wooden applicator and then rotating on a Junior Orbit Shaker (Lab-line Instruments, Inc., Melrose Park, IL) for 5 min. Directigen test was performed according to the manufacturer's instructions.

Counterimmunoelectrophoresis (CIE). In performing CIE, a buffered diffusion medium is used into which a series of opposing wells have been punched for samples containing either antigens or antibodies. At the pH usually employed (weakly alkaline), the antigens generally will be negatively charged and will migrate towards the anode on application of electric current through the diffusion medium. Antibodies, although weakly negative or neutral, will be swept towards the cathode by a

Comparison of counterimmunoelectrophoresis (CIE), latex agglutination (LA) and staphylococcal coagglutination (COAG) in pneumococcal capsular polysaccharide (PCP) antigen detection

Technique	Least amounts of (PCP) antigen detected (μg/ml) Pneumococcal types			
	3	9	7	14
CIE	0.09	0.78	25	50
LA-Directigen	0.0055	0.045	1.56	0.39
COAG-Phadebact	0.045	0.78	6.25	1.56
COAG-Pansorbin	0.19	0.78	3.1	3.1
COAG-Cowan 1	0.78	0.78	3.1	3.1