results, it can be concluded that the antisera prepared using antigen extracts from the collection reference strains are specific at the species or subspecies level when used in ODP or IEF systems.

In order to test the antisera against wild-type strains, we analyzed 8 *Aeromonas* strains using the immunochemical techniques described. These strains were isolated from fishes and identified as *A. hydrophila* subsp. *hydrophila* according to the biochemical characters specified by Bergey[11]. The table, B summarizes the results found; all the wild type strains reacted with the *A. hydrophila* subsp. *hydrophila* antiserum and did not react with the other antisera. The ODP technique gave 2 precipitation bands, whereas the IEF gave 4 bands similar to those found using the reference strain extract. Figure 2, B shows an example of such a reaction (band No. 2 is difficult to see because it is close to the trough). We can thus conclude that the antisera we prepared can be used to identify specifically *Aeromonas* species or subspecies, particularly the *A. hydrophila* subsp. *hydrophila* strains.

Two final remarks can be made. The first one is related to the taxonomic grouping of the species *A. hydrophila* into the subspecies *hydrophila* and *anaerogenes*; since our results show that these subspecies seem to be not antigenically related, one may wonder if they really ought to belong to the same species. The other remark concerns the antigenicity of the *Aeromonas hydrophila* strains isolated from fish in a pathological condition. Preliminary ODP and IEF assays performed with antisera prepared from these wild-type isolates show the presence of additional precipitation bands when compared to the results obtained using the antiserum specific for the reference strain. This may indicate the presence of external proteins involved in the pathogenic process of these bacteria; in the absence of the selective conditions encountered during the process of parasitizing a host, these antigens would disappear and would thus not be detectable in collection strains subcultured in laboratory media. Experiments are in progress to investigate the role of these antigens in the virulence of *Aeromonas hydrophila* (Lallier et al.[12] and this laboratory).

1 Present address and to whom reprint requests should be addressed: Istituto Cantonale Batteriologico, via Ospedale 6, CH-6904 Lugano, Switzerland.
2 Skinner, F.A., and Sherwan, J.M., *Aeromonas salmonicida* in Aquatic microbiology. Academic Press, London/New York 1977.
3 Bullock, G.L., and Stuckey, H.H., Prog. Fish. Cult. *37* (1975) 237.
4 Jensen, N.J., and Larsen, J.L., in: Fish diseases, p. 87. Ed. W. Ahne. Springer, Berlin/Heidelberg/New York 1980.
5 Sniezko, S.F., Mar. Fish. Rev. *40* (1978) 65.
6 Seidler, R.J., Allen, D.A., Lockmann, H., Colwell, R.R., Joseph, S.W., and Daily, O.P., Appl. Envir. Microbiol. *39* (1980) 1010.
7 Joseph, S.W., Daily, O.P., Hunt, W.S., Seidler, R.J., Allen, D.A., and Colwell, R.R., J. clin. Microbiol. *10* (1979) 46.
8 de Meuron, P.A., and Peduzzi, R., Zentbl. VetMed. B *26* (1979) 153.
9 Ouchterlony, O., Prog. Allergy *6* (1962) 30.
10 Scheidegger, J.J., Int. Archs Allergy appl. Immun. *7* (1955) 103.
11 Bergey's Manual of determinative bacteriology, 8th edn. Williams & Wilkins, Baltimore 1974.
12 Lallier, R., Mittal, K.R., Lalonde, G., and Olivier, G., in: Developments in biological standardization, vol. 49, p. 119. Karger, Basel 1981.

0014-4754/83/080924-03$1.50 + 0.20/0


## A new method for detection of anti-zona activity in human sera using latex agglutination reaction

Y. Noda, I. Yamada, S. Hayashi, I. Takai and E. Watanabe

*Department of Obstetrics and Gynecology, School of Medicine, Kyoto University, 54 Shyogoin Kawahara-cho, Sakyo-ku, Kyoto 606 (Japan), and Daigo-Watanabe Womens Hospital, Takahata-cho, Daigo, Kyoto 601-13 (Japan), November 5, 1983*

*Summary.* A slide latex test for detection of anti-zona pellucida activity in human sera was developed using a latex agglutination reaction. The latex reagent, made of polystyrene particles coated with solubilizing zona antigen(s), was found to give results comparable in sensitivity as well as specificity to those of the indirect immunofluorescence method as tested with anti-pig-zona antiserum and with human sera. Thus, the slide latex test was judged to be adequate for use instead of the indirect immunofluorescence technique.

Because of the presence of zona-specific antigen[1,2] and the antifertility activity of anti-zona antibody[3], increased attention has been focused on the zona pellucida as one of the target structures for immunological regulation of fertility[4]. Though the presence of autoantibodies to the zona has been postulated as a potential etiologic factor for infertility in women[5,6], this hypothesis has recently been disputed on the basis of both the reactivity of anti-zona antibodies to various tissues other than the zona[7], and the distribution of serological anti-zona activity in males, females and children[8]. One of the reasons for the controversy seems to be the method employed for detection of anti-zona activity in human sera; all the work published so far has employed the indirect immunofluorescence technique using pig oocytes as the target. Although a theoretical basis was provided by the apparent presence of common antigen(s) in human and pig zonae, the indirect immunofluorescence technique could not be considered entirely satisfactory because of difficulty in quantitation and its time-consuming nature. Therefore, it is indispensable to develop a simple and sensitive method for the characterization of anti-zona activity in human sera. In our laboratory, a method for preparation of pig zona pellucida has been developed[9] and highly purified pig zona components have been successfully isolated[10]. This preliminary work made it possible for us to develop a new method. Details of the experimental conditions for preparation of the latex reagent and quality assessment of the sensitivity and specificity of the reaction are described in this paper.

*Materials and methods.* The lyophilized pig zona material

Table 1. Parallel titration of anti-zona pellucida antibody by the slide latex and the indirect immunofluorescence tests

| | | Dilution of the anti-serum | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 1:2 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 | 1:512 | 1:1024 |
| Slide latex test[c] | Porcine[a] | + | + | + | + | + | + | + | + | + | + | – |
| | Control[b] | – | – | – | – | – | – | – | – | – | – | – |
| Indirect IFT[d] | Porcine | + | + | + | + | + | + | + | + | + | + | – |
| | Control | – | – | – | – | – | – | – | – | – | – | – |

[a]Rabbit antiserum to porcine zona pellucida. [b]Normal rabbit serum. [c]Slide latex agglutination test. [d]Inderect immunofluorescence test using porcing oocytes as a target.

Table 2. Anti-pig zona antibody detected by latex agglutination and indirect immunofluorescence

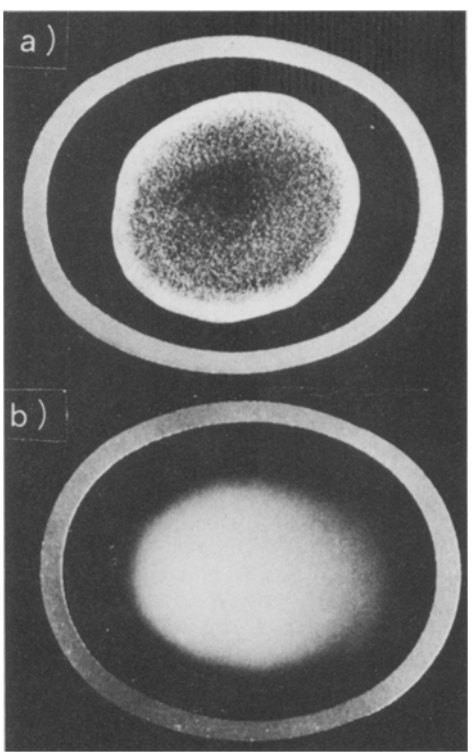
| | Titers of positive latex agglutination | | | | No. of | Immunofluorescence | |
|---|---|---|---|---|---|---|---|
| | 1:4 | 1:2 | 1:1 | (–) | sera tested | (–) | (+) |
| Infertile | 2 | 1 | 2 | 20 | 25 | 20 | 5 |
| Pregnant[a] | 0 | 1 | 5 | 29 | 35 | 29 | 6 |
| Unmarried[b] | 1 | 2 | 9 | 8 | 20 | 8 | 12 |
| Men[c] | 1 | 0 | 3 | 6 | 10 | 6 | 4 |
| Aged[d] | 1 | 2 | 2 | 8 | 13 | 8 | 5 |
| Total | 5 | 6 | 21 | 71 | 103 | 71 | 32 |

[a]Between 13 and 38 weeks' gestation. [b]Unmarried women aged 18 to 21 years old. [c]Fertile men aged 25 to 40 years old. [d]Over 40 years old.

(17.4 mg) was dissolved in 0.1 M Na-borate pH 10.0 at 60 °C for 3 h, then centrifuged (12000 × g, 30 min), concentrated (8.8 OD at 280 nm in 5.2 ml), and filtered by Sephadex G-100 column chromatography. The solubilization efficiency and the total recovery of the column procedure were 94% and 98%, respectively; 79% of the solubilized zona material (OD 6.9 at 280 nm, 10.2 mg protein) was recovered in the combined main fractions of 51–61, which were used for the preparation of the latex reagent or as the immunogen.

In the preparation of the latex reagent, 0.23 ml of polystyrene particles (9.6% w/v, 0.47 µm in diameter, Sekisui latex for diagnostics, Sekisui Chemicals, Osaka) were mixed with 3.77 ml of 20 mM Na-phosphate pH 7.0 containing 0.5 mg of solubilized pig zona components which had been dialyzed against 20 mM Na-phosphate pH 7.0. The mixture was stirred for 4 h at room temperature, and after washing twice with phosphate buffer, the polystyrene particles coated with the solubilized zona components were recovered in a tube and kept stirring for 1.5 h at 25 °C in phosphate buffer containing 0.2% bovine serum albumin (Sigma, crystal) and 0.001% sodium dodecyl sulfate. At this stage the self-agglutinating tendency of the particles was diminished. The particles were finally recovered in 1.1 ml phosphate buffer containing 0.1% $NaN_3$ and 0.8% sucrose. For the latex agglutination reaction, 30 µl serum and 10 µl latex were mixed together on the glass plate. A clear agglutination was observed within 3 min after the mixing if positive (fig.). On preparation of anti-pig zona rabbit serum, the immunogen (0.5 mg protein) was emulsified with an equal volume of Freund's complete adjuvant and used for immunizing a male New Zealand white rabbit. The rabbit was boosted every 2 weeks and bled 14 days after the 5th booster injection. Normal rabbit serum was obtained from the pre-immune rabbit. Human serum samples were obtained from volunteers or outpatients. The sera were inactivated and stored at −20 °C until use. Indirect immunofluorescence was performed employing porcine oocytes as the target. Porcine oocytes were incubated with sera for 30 min at 37 °C. After washing with phosphate buffered saline (PBS), the oocytes were allowed to react for 30 min at 37 °C with rabbit antiserum to human IgG (Miles-Yeda Ltd, Israel) or pig antiserum to rabbit IgG (DACO-immunoglobulin, Copenhagen) labeled with fluorescein isothiocyanate. The oocytes were then washed with 5 ml PBS for 60 min at 37 °C and the reaction was observed under a fluorescence microscope (Olympus Type BH-REL) and graded positive (+) or negative (−). Titers of sera were expressed as the maximum dilution which gave a positive reaction with these 2 methods.

*Results and discussion.* The sensitivity and specificity of the latex reagent were compared to those of the indirect




Latex agglutination reaction at room temperature; *a*, positive, *b* negative.

immunofluorescence using anti-pig zona rabbit serum, normal rabbit serum and human serum samples. The same antibody titer (1:512) could be detected with the standard antiserum by these 2 methods and no reaction was found with normal rabbit serum (table 1). Of 103 sera from men and women tested using latex reagent, 32 (31%) gave positive reactions regardless of the sources of sera, showing a maximum titer of 1:4 (table 2). All the positive sera in latex agglutination showed a positive reaction on the outer surface of the porcine zonae as determined by indirect immunofluorescence, whereas negative sera did not. No serum sample gave a contradictory reaction with these 2 methods. The reactivity of the latex reagent did not change for more than a month when stored at 4 °C.

These results indicated that the slide latex test investigated in this study is comparable in sensitivity and specificity to the method using indirect immunofluorescence. Moreover, considering the simplicity of the procedure, as well as the short time of the reaction, this new method should be valuable as a simple screening method for the detection of anti-zona pellucida activity in human sera instead of indirect immunofluorescence.

It has been reported that a common antigen is shared by human and porcine zonae[11], but differences in antigenic composition of human and pig zona have also been suggested[12]. Considering these reported differences, as well as the possible presence of hetero-agglutinin in human sera, the detection of anti-pig zona activity in this study does not necessarily indicate the presence of anti-zona antibody. Further characterization of anti-zona activity in human sera is now being done in our laboratory using the slide latex test.

1 Ownby, C.L., and Shivers, C.A., Biol. Reprod. *6* (1972) 310.
2 Sacco, A.G., and Shivers, C.A., J. Reprod. Fert. *32* (1973) 415.
3 Yanagimachi, R., Winkelhake, J., and Nicolson, G.L., Proc. natl Acad. Sci. USA *73* (1976) 2405.
4 Shivers, C.A., in: Immunological approaches to fertility control, p.223. Ed. E.D. Deczfalusy. Karolinska Institute, Stockholm 1974.
5 Shivers, C.A., and Dunbar, B.S., Science *197* (1977) 1082.
6 Mori, T., Nishimoto, T., Kohda, H., Takai, I., Nishimura, T., and Oikawa, T., Fert. Steril. *32* (1979) 67.
7 Nayudu, P.L., Freemann, L.E., and Trounson, A.O., J. Reprod. Fert. *65* (1982) 77.
8 Dietl, J., Knop, G., and Mettler, L., J. Reprod. Immun. *4* (1982) 123.
9 Noda, Y., Mori, T., Takai, I., Kohda, H., and Nishimura, T., J. Reprod. Immun. *3* (1981) 147.
10 Noda, Y., Kohda, H., Takai, I., Hayashi, S., Shimada, H., Mori, T., and Tojo, S., J. Reprod. Immun. *5* (1983) 161.
11 Sacco, A.G., Biol. Reprod. *16* (1977) 164.
12 Takai, I., Mori, T., Noda, Y., and Nishimura, T., J. Reprod. Fert. *61* (1981) 19.

0014-4754/83/080926-03$1.50 + 0.20/0


## Bioluminescence assay of calcium with a liquid scintillation counter

C. Oveby, R. Kant and S. Batra[1]

*Leo Research Laboratories, AB Leo, Box 941, S-251 09 Helsingborg (Sweden), August 23, 1982*

*Summary.* The luminescence produced by Ca aequorin reaction in dilute solutions decays exponentially over a relatively prolonged period of time. The concentration of total Ca and of free Ca in Ca-EGTA buffers could be determined by measuring the decay of luminescence in a liquid scintillation counter. The method is also suitable for determining total Ca concentration in small tissue samples.

A number of reports in the past have drawn attention to the possibilities offered by use of scintillation counters for the measurement of bioluminescence produced by firefly extract in the presence of ATP[2-4]. With the commercial availability of aequorin, a calcium-sensitive luminescent protein, we decided to explore the possibility of measuring calcium concentration using a liquid scintillation counter. Conditions are described for the measurement of total calcium and, under certain conditions, of free calcium in calcium-EGTA system using aequorin. The method is also suitable for determining total calcium concentration in small tissue samples.

*Materials and methods.* Calcium aequorin reaction was carried out in a buffer solution containing 2 mM each NaCl, KCl, $MgSO_4$ and 10 mM of Tris, pH 7.0. This solution which was made from ultra pure chemicals was passed through a Chelex column to remove contaminating calcium. Preliminary experiments showed that without Chelex treatment the blank values were about 10 times higher than those obtained with Chelex-treated solutions[5]. 10 ml of ice-cold buffer solution were pipetted into polyethylene bottles (Packard) kept on ice. 50 or 100 µl of aequorin solution (1 mg aequorin, Type 3, dissolved in 1 ml $H_2O$) were added and the contents shaken. 100 µl of $CaCl_2$ standards (0–40 nmoles) were then added to different bottles and vortexed for 10 sec. After 15 or 30 sec of waiting, samples were counted repeatedly for 10 0.1-min intervals in a Packard Tri-Carb liquid scintillation counter in a tritium channel. Total tissue calcium concentration in the uterus and aorta was determined after removing the adhering fat and connective tissues. Tissue pieces weighing about 100 mg were taken, weighed and thereafter dried for 16–24 h at 100 °C. After determining the dry weight, tissues were digested by heating at 200 °C with 5 drops of concentrated $HClO_4$ and $HNO_3$ (1:1). The tissues were completely ashed after 24 h. The ash was dissolved in 2 ml of water and 100 µl of the sample as that of the standard was used for Ca assay.

Total calcium in rabbit uterus and aorta

| Tissue | Total Ca (mmoles/kg) Wet weight | Dry weight |
|---|---|---|
| Uterus | 1.35 ± 0.14 | 10.16 ± 1.17 |
| Aorta | 3.32 ± 0.36 | 12.87 ± 1.66 |

Values are means ± SE of 4 determinations.