injected only 1 µl of 5–10 mouse LD_{50} TT), but signs of neuronal damage attributable to the toxin were still lacking in our animals.

We conclude from these contradictory effects in KA- and TT-treated animals that an explanation of the widespread neuronal damage following KA-injections by an anterograde propagation of epileptic activity[7,18] is not supported by our data. Duration of seizure activity per se can account for neither the many damaged loci in KA-treated rats, nor the lack of damage in TT-treated rats. Dam et al.[19] have shown with electrical stimulation of hippocampal neurons that even a number of up to 140 induced seizure attacks each of about 50-sec duration may fail to result in neuronal loss in the hippocampus.

Seizure activity may accompany the decay of neuronal tissue in widespread loci of the brain following injections of KA, but apparently it is not its primary cause. This must be sought in the chemical properties of KA itself and in the interaction of KA with specific neurotransmitters (glutamate, aspartate)[9].

1 We thank Behring AG (Marburg/Lahn, FRG) for the generous gift of tetanus toxin. D. Emmans deserves thanks for improvement of the English text. Research was supported by grant Ma 795/3 of the Deutsche Forschungsgemeinschaft.

2 Author for correspondence and reprint requests.

3 Divac, I., Markowitsch, H.J., and Pritzel, M., Brain Res. *151* (1978) 523.
4 Guldin, W.O., and Markowitsch, H.J., J. Neurosci. Meth. *5* (1982) 83.
5 Kessler, J., and Markowitsch, H.J., Brain Res. Bull. *8* (1982) 439.
6 Ben-Ari, Y., in: Glutamate as a neurotransmitter, p.385. Ed. G. DiChiara. Raven Press, New York 1981.
7 Ben-Ari, Y., Tremblay, E., and Ottersen, O.P., Neuroscience *5* (1980) 515.
8 Ben-Ari, Y., Tremblay, E., Riche, D., Ghilini, G., and Naquet, R., Neuroscience *6* (1981) 1361.
9 Heggli, D.E., and Malthe-Sørensen, D., Neuroscience *7* (1982) 1257.
10 McGeer, P.L., and McGeer, E.G., in: Advances in cytopharmacology, vol.3, p.437. Eds B. Ceccarelli and F. Clementi. Raven Press, New York 1979.
11 Schwob, J.E., Fuller, T., Price, J.L., and Olney, J.W., Neuroscience *5* (1980) 991.
12 Zaczek, R., Nelson, M., and Coyle, J.T., Neuropharmacology *20* (1981) 183.
13 Zaczek, R., Simonton, S., and Coyle, J.T., J. Neuropath. exp. Neurol. *39* (1980) 245.
14 Nadler, J.V., and Cuthbertson, G.J., Brain Res. *195* (1980) 47.
15 Guldin, W.O., and Markowitsch, H.J., Brain Res. *225* (1981) 446.
16 Mellanby, J., George, G., Robinson, A., and Thompson, P., J. Neurol. Neurosurg. Psychiat. *40* (1977) 404.
17 Mellanby, J., Strawbridge, P., George, G., Rands, G., Stroud, C., and Thompson, P., J. Neurol. Neurosurg. Psychiat. *44* (1981) 1084.
18 Smialowski, A., and Smialowski, M., Neuroscience *7* (1982) 2519.
19 Dam, A.M., Hertz, M., and Bolwig, T.G., Brain Res. *193* (1980) 268.

0014-4754/83/080922-03$1.50+0.20/0

Investigation of *Aeromonas* isolated from water; a serological study using Ouchterlony and immunoelectrophoresis techniques

R. Peduzzi[1], P. A. de Meuron and E. Grimaldi

Swiss Federal Institute for Water Resources and Water Pollution Control (EAWAG), CH-8600 Dübendorf (Switzerland), Swiss Federal Institute of Technology (ETH), CH-8092 Zürich (Switzerland), Service vétérinaire Cantonal et Institut Galli-Valerio, CH-1005 Lausanne (Switzerland), and CNR, Istituto Italiano di Idrobiologia, I-28048 Pallanza (Italy), *February 10, 1982*

Summary. We have shown that immunoelectrophoresis and the Ouchterlony double diffusion technique are valuable assays for the identification of *Aeromonas* species. Serological correlations have been found between the antigenic extracts originated from a collection reference strains of *Aeromonas hydrophila* subsp. *hydrophila* and those originated from wild type water isolated.

To the genus *Aeromonas* belong important fish pathogens responsible for several communicable diseases such as furunculosis; thus, these bacteria may be of economic significance in commercial fish farming[2-4]. Moreover, from an epidemiological point of view, they can be considered as pollution indicator organisms for aquatic environments[5-7]. For these reasons, methods should be developed to allow rapid and reliable differentiation among these bacteria, at the species and possibly subspecies levels. Two serology assays have already been examined by two of us; comparisons between morphological, physiological, biochemical characters and slide or tube agglutination tests have shown that, in the conditions used, these immunochemical methods were not reliable for species identification purposes, mainly because of their lack of specifity[8]. To determine whether the data given by serology can be improved, we assayed two other techniques (Ouchterlony immunodiffusion and immunoelectrophoresis) using soluble antigen extracts. The results are discussed in this paper.

Materials and methods. Bacterial strains. Reference *Aeromonas* strains were obtained from the American Type Culture Collection (ATCC) and the National Collection of Marine Bacteria (NCMB; Aberdeen, Scotland). These were: *A. salmonicida* subsp. *salmonicida* NCMB 1102, *A. hydrophila* subsp. *hydrophila* NCMB 86, *A. hydrophila* subsp. *anaerogenes* ATCC 15467, *A. punctata* subsp. *punctata* NCMB 74.

Wild type *Aeromonas* strains were isolated from fishes; some of them have been used in our previous investigation[8]: 3617/74, 7535/73, 1561/A1/4, 2535/73, 5816/75, 3569/74, 8430/75, 2437/76.

Growth conditions. Aeromonas strains were isolated on Infusion Agar (BBL 11037) containing 5% sheep blood or on Bromthymol Blue Lactose Agar (BBL 93961). After their isolation, the strains were cultured on DST Agar (Oxoid CM 261), on Furunculosis Agar (Difco 0350-01), or on PPLO Agar (Difco 0412-01). Plates were incubated 24 h at room temperature or at 30 °C.

Ouchterlony and immunoelectrophoresis reactions between reference-collection (A) and wild type (B) *Aeromonas* strains and sera specific for the reference-collection strains

Antigens	Serum specific for antigen: 1	2	3	4
A				
1. *A. salmonicida* subsp. *salmonicida* NCMB 1102	+	−	−	−
2. *A. hydrophila* subsp. *hydrophila* NCMB 86	−	+	−	−
3. *A. hydrophila* subsp. *anaerogenes* ATCC 15467	−	−	+	−
4. *A. punctata* subsp. *punctata* NCMB 74	−	−	−	+
B				
5. *A. hydrophila* subsp. *hydrophila* 7935	−	+	−	−
6. *A. hydrophila* subsp. *hydrophila* 3617	−	+	−	−
7. *A. hydrophila* subsp. *hydrophila* 3535	−	+	−	−
8. *A. hydrophila* subsp. *hydrophila* 8430	−	+	−	−
9. *A. hydrophila* subsp. *hydrophila* 3569	−	+	−	−
10. *A. hydrophila* subsp. *hydrophila* 2437	−	+	−	−
11. *A. hydrophila* subsp. *hydrophila* 156 1A	−	+	−	−
12. *A. hydrophila* subsp. *hydrophila* 5816	−	+	−	−

Antigenic extracts and antisera. Antigenic extracts: bacterial cultures grown for 24 h on DST agar were suspended in 15 ml 0.9% NaCl, 0.5% formalin and centrifuged 10 min at 10,000 × g and 4 °C. The pellet was resuspended in 1 ml water and 2 ml of buffer (Tris-HCl 0.033 M, pH 7.1; EDTA 0.1 mM; lysozyme 100 µg/ml) were then added; this suspension was centrifuged (10,000 × g, 4 °C, 10 min) and the supernatant containing the soluble antigens was used for the immunochemical assays. Antisera: bacterial cultures grown for 12–24 h on DST or PPLO agar were suspended in 0.9% NaCl containing 1% formalin. Cells were washed twice in 0.9% NaCl. The final suspension was used to produce complete antisera OK (H).

To produce antisera, hens were injected i.v. (2 ml) every 48 h for 12 days; the blood was collected 7 days after the last injection.

Serology methods. Double diffusion precipitation: this was carried out according to Ouchterlony[9]. Antigens were added to 6 external wells (see fig. 1), at a distance of 9 mm from the central well containing the antiserum. The amount of reagent in each well varied between 70 and 100 µl. Diffusion time was 48 h.

Immunoelectrophoresis: this was performed according to Scheidegger's micromethod[10] on 76 × 26 mm glass slides covered with a 1 mm thick agarose layer (1% in sodium-diethylbarbiturate, 0.05 M, pH 8.2). After electrophoresis (45 min, 210 V) the antiserum was added and the reagents were allowed to diffuse for 48 h. The slides were then rinsed in NaCl 0.9%, dried, stained with thiazine red or Coomassie blue, and destained.

Results and discussion. We first assayed the reference strains against their specific antisera. The results found using both the Ouchterlony double diffusion precipitation technique (ODP) and the immunoelectrophoresis (IEF) are summarized in the table, A; each antiserum reacted with its specific antigen extract and did not react with the extracts from different species or subspecies. When positive, the ODP technique gave 2 precipitation lines; this can be seen in figure 1, where the antiserum specific for *A. salmonicida* shows 2 bands of precipitate with its homologous antigen extract and no bands with extracts from *A. hydrophila* subsp. *hydrophila*, *A. hydrophila* subsp. *anaerogenes* and *A. punctata* subsp. *punctata*. The IEF technique usually showed 3 or 4 bands for the homologous reactions and no bands for the heterologous ones. This can be seen in figure 2,A, where the antiserum against *A. hydrophila* subsp. *hydrophila* gives 4 precipitation lines (arrows 1–4) when reacting with its specific antigen extract. From these

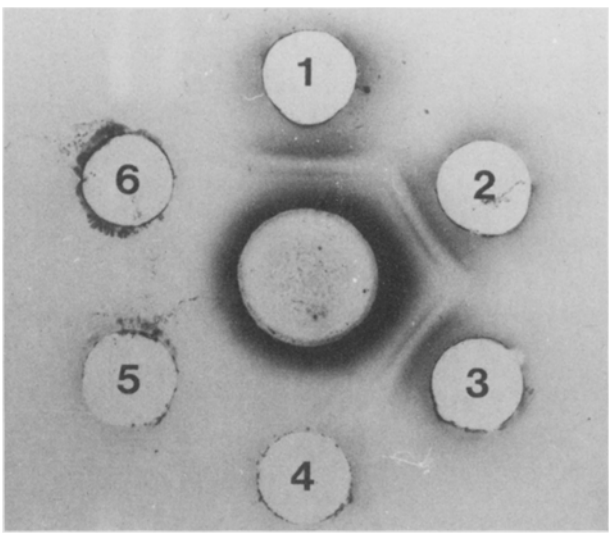

Figure 1. Ouchterlony double precipitation technique. Two precipitation bands are visible between the antiserum specific for *A. salmonicida* (central well) and its homologous antigenic extracts (wells 1, 2, 3); no bands are detectable with the antigenic extracts from *A. hydrophila* subsp. *hydrophila* (well 4), *A. hydrophila* subsp. *anaerogenes* (well 5), and *A. punctata* subsp. *punctata* (well 6).

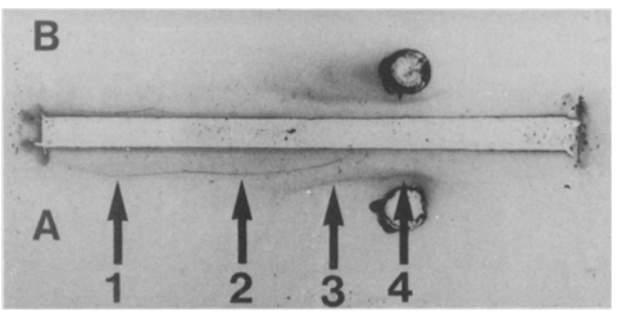

Figure 2. Immunoelectrophoresis technique. In the trough is the serum specific for the reference-collection strain *A. hydrophila* subsp. *hydrophila*. *A* Antigenic extract from a wild type isolate of *A. hydrophila* subsp. *hydrophila*. *B* Antigenic extract from the homologous reference strain. In B, band No. 2 is not clearly visible because it is too close to the trough. The anode was at the left.

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