

Experimental vaccine against *Pseudomonas pseudomallei* infections in captive cetaceans

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ABSTRACT: The antibody response to an experimental acellular *Pseudomonas pseudomallei* vaccine was evaluated by Enzyme Immunoassay in captive cetaceans at Ocean Park, Hong Kong. The vaccine preparation consisted of a protein-polysaccharide (1:3) mixture with trace nucleic acids and no 2-keto-3-deoxyoctonate. An initial dosage of 500 µg carbohydrate for newly arrived dolphins *Tursiops aduncus* and false killer whales *Pseudorca crassidens* in 1987 and 1 mg carbohydrate for other long-term resident animals (1983 to 1988) followed by 500 µg boosters at ca 6 to 10 wk intervals for all animals maintained high specific antibody levels. Mortality due to melioidosis was reduced over a 5 yr period from 45 % to less than 1 %.

INTRODUCTION

Pseudomonas pseudomallei is the etiologic agent of melioidosis, a disease principally of rodents but also occasionally of humans. The microorganism is indigenous in the soil and water of southeast Asia and infections in humans occur via the respiratory tract or through contaminated wounds and abrasions (Vedros 1988).

Captive animals appear to be susceptible to melioidosis in southeast Asia, and this has been particularly true of captive cetaceans in Hong Kong (Liong et al. 1985). The highest mortality occurs during the rainy season (May–Oct). During this period considerable amounts of soil are washed down from mountains into bays adjacent to Hong Kong island. Some of these bays are used as a source of water for the Ocean Park cetacean pools. Further, the animals are under stress due to high winds and heavy rainfall.

In 1983 a vaccine trial was initiated using an acellular preparation of *Pseudomonas pseudomallei*. This report describes the antibody response to the experimental vaccine in 5 species of cetaceans. Antibody levels are presented for 1 group of animals during the rainy season in 1987, 1 group during the dry, fall season (1987 to 1988), and animals representative of those resident during the 5 yr vaccine trial.

METHODS AND MATERIALS

Microorganisms. Strain SLT *Pseudomonas pseudomallei* was isolated in 1982 from lung tissue in a fatal melioidosis case (dolphin *Tursiops aduncus*) on Brain Heart Infusion agar (BHI; Difco Co., Detroit, MI, USA) containing 1:200 000 crystal violet and 1 % gentamycin. This strain was used for preparation of the vaccine and antigen in the Enzyme Immunoassay (EIA) in this study although 19 other strains were isolated from tissues in fatal melioidosis cases of 1 stellar sea lion *Eumetopias jubata*, 1 northern fur seal *Callorhinus ursinus*, 11 *Tursiops aduncus*, and 6 *Tursiops gilli*. All strains belonged to serological Type 1. Initial biochemical and morphological criteria for identification were essentially as described by Dodin & Galimond (1976) and listed in Table 1. The final step in the identification was agglutination with a specific anti-Type I *P. pseudomallei* serum kindly supplied by Dr Paul Martin of the Institut Pasteur. All strains were maintained in lyophile form at 4 °C in their third passage from the source.

Vaccine preparation. The acellular vaccine was prepared by a modification of the method for isolating surface components of *Neisseria meningitidis* as described by Apicella (1976). Strain SLT was grown overnight at 36 °C on BHI agar plates containing 1 %

Table 1. Morphological/biochemical reactions used to identify *Pseudomonas pseudomallei*

Test	Reaction
Staining	Gram negative rod; bipolar staining
Motility	Positive
Nitrate reduction	Positive to nitrite
Growth on McConkey's agar	Positive
Urease	Negative
Simmon's citrate	Positive
Gelatin liquifaction	Positive
Arginine dihydrolase	Positive
Poly- β -hydroxybutyrate accumulation	Positive
OF-Maltose	Oxidizer positive; fermentation negative

glycerol. Bacteria were harvested with BHI broth and inoculated into 12 l of BHI broth containing 1 % glycerol. Final concentration was approximately 10^6 bacteria ml^{-1} . After 48 h stationary growth (36 °C) the pellicle was disrupted by shaking with sterile glass beads (10 to 12 mm diameter, 70 g l^{-1}) for 72 h at room temperature. Bacteria and beads were removed by centrifugation ($7000 \times g$, 20 min) and formalin added to the supernatant (0.1 % final concentration). After 48 h at 4 °C, dry ammonium sulfate (540 g l^{-1}) was slowly added, stirred for 24 h (4 °C) and precipitate was solubilized in distilled water and extensively dialysed against distilled water until ammonium ions were removed. The dialysate was concentrated 10-fold (Amicon, PM-30 membrane) and adjusted to pH 11 with 1.0 N NaOH. After 1 h at room temperature the pH was adjusted to 7.0 with 0.1 N HCl. The precipitate was removed by centrifugation ($20\,000 \times g$, 30 min) and the supernatant, after equilibration, added to a column of Sepharose Cl-4B gel (Pharmacia, $100 \times 2.5 \text{ cm}$). The fractions were eluted with 0.02 M phosphate buffer, pH 6.5. The carbohydrate containing fractions were pooled, ethanol added to a final concentration of 25 % and precipitate removed after 2 h stirring at room temperature. The alcohol was removed from the supernatant under vacuum. The final vaccine was lyophilized, stored at 4 °C and when needed reconstituted with sterile distilled water. An immunizing dose of the vaccine (1 mg of carbohydrate) contained 259 μg of protein, trace nucleic acid, and no 2-keto-3-deoxyoctonate.

IgG isolation. The IgG of the pooled sera from 6 *Tursiops aduncus* was isolated through a column of DEAE AFFI GEL BLUE as described by the manufacturer (Biorad, Richmond, CA, USA, 1988).

Rabbit antisera. Rabbit anti-IgG was produced by subcutaneous (s.c.) injection of 1.0 mg IgG mixed in equal volume of Complete Freund's Adjuvant (Difco Co., Detroit, MI, USA) followed by a booster 3 wk later of 1.0 mg IgG in Incomplete Adjuvant (s.c.). Rabbits were bled 7 d later.

Rabbit antisera to the vaccine antigen was produced

by a similar procedure as above using 2.0 mg carbohydrate. A third booster of 3.0 mg carbohydrate without adjuvant was given intravenously (i.v.) 3 wk later and the rabbits bled 10 d later. All sera were stored at -20 °C.

Assay of vaccine in hamsters. Three groups of 6 hamsters each (av. wt 126 g) were injected subcutaneously (s.c.) in 0.2 ml aliquots with 10, 50, and 100 μg of vaccine (carbohydrate) respectively and boosted 3 wk later with similar dosages s.c. Controls received 0.2 ml saline s.c. The animals were challenged 10 d later with 20 Minimal Lethal Doses (MLD) of Strain SLT *Pseudomonas pseudomallei* and death recorded over a 7 d period. Pure cultures of *P. pseudomallei* were isolated from the spleen, liver, and blood of those hamsters that died.

Enzyme immunoassay (EIA). Procedures and reagents were as previously described (Suer et al. 1988). The vaccine was solubilized in bicarbonate buffer (pH 9.6) and the optimum concentration (125 ng ml^{-1} carbohydrate) to coat the EIA plates determined by block titration against rabbit antisera to the vaccine. Optimum serum dilution (1:200) was determined by block titration of the pooled sera of 4 *Tursiops aduncus* that were positive (optical density, O. D. = > 1.0) by the EIA to the vaccine antigen and the pooled sera of 2 *T. gilli* that were negative (O. D. = < 0.2). Both positive and negative serum controls were included in every test.

Optimum dilutions of rabbit anti-dolphin IgG and goat anti-rabbit IgG (H&L chains) horseradish peroxidase conjugate (Zymed Labs., San Francisco, CA, USA) were determined using $10 \mu\text{g ml}^{-1}$ of purified dolphin IgG.

Cetacean immunization. One group of 9 false killer whales *Pseudorca crassidens* that arrived in April 1987 were injected intramuscularly (i.m.) with 500 μg carbohydrate (1.8 to $2.2 \mu\text{g kg}^{-1}$) and boosted with 500 μg carbohydrate i.m. in June and August except as noted. Another group of 8 bottlenose dolphins *Tursiops aduncus* that arrived in November 1987 were injected i.m. with 500 μg carbohydrate (2.3 to $3 \mu\text{g kg}^{-1}$) and boosted i.m. with 500 μg carbohydrate in December

Four *Tursiops gilli* that arrived in March 1986 were

injected i.m. with 1 mg carbohydrate (1.7 to $3 \mu\text{g kg}^{-1}$) and boosted i.m. with $500 \mu\text{g}$ carbohydrate in mid-August.

The other cetaceans (6 *Tursiops aduncus*, 1 *Lagarenchus obliquidens*, 1 *Orcinus orca*) resident in Ocean Park from 1983 to 1988 were injected i.m. yearly with 1 mg carbohydrate in the spring (Mar–Apr) and boosted i.m. with $500 \mu\text{g}$ carbohydrate in July or August and then again in either September, October or November.

Chemical analyses. Proteins were determined as described by Lowry et al. (1951) using bovine serum albumin as standard. Carbohydrates were determined as described by Dubois et al. (1956), using glucose as standard. Nucleic acids were determined at OD_{260} and OD_{280} in a Model 552 Perkins-Elmer scanning spectrophotometer. Determination of 2-keto-3-deoxyoctonate was by the thiobarbituric acid assay as described by Osborne (1963).

RESULTS

Vaccine

The yield of vaccine averaged 22 mg carbohydrate per liter of culture. Yield per liter was less (3 to 12 mg l^{-1}) in 2 experiments where cultures were not stationary, but incubated on a gyratory shaker (120 rpm). Further detailed chemical analysis of the vaccine components is in progress.

At dosages above $20 \mu\text{g}$ carbohydrate (initial injection plus 1 booster) the vaccine protected 10 out of 12 hamsters challenged with 20 MLD of strain SLT *Pseudomonas pseudomallei* (Table 2). Protection did not increase with immunizations above $50 \mu\text{g}$ carbohydrate (total).

Immune response in false killer whales

The EIA antibody levels in 3 of 9 false killer whales *Pseudorca crassidens* from their arrival at Ocean Park

Table 2. Protective effect of *Pseudomonas pseudomallei* vaccine in hamsters. Vaccine lot #86-11. Animals boosted with same dosage 3 wk after primary immunization and challenged 10 d later with 20 MLD. Death observed over 7d period

Group ^a	Vaccine dosage (μg)	Number of animals dead/survivors
1	10	5/1
2	50	1/5
3	100	1/5
4	Saline	6/0

^a Six animals per group

in April 1987 through September 1987 are shown in Fig. 1a. The booster injections of $500 \mu\text{g}$ carbohydrate in June and August maintained antibody levels throughout the rainy season. Four other false killer had similar antibody levels. Two other individuals had relatively low antibody levels in June, consequently the second booster in August was increased to 1 mg carbohydrate. Antibody levels rose similarly to those in the other 7 false killer whales (Fig. 1b).

Immune response in dolphins

Representative EIA antibody levels in 3 of 8 *Tursiops aduncus* from their arrival on 3 November 1987 through 7 January 1988 are shown in Fig. 2. The range of EIA values for all 8 individuals prior to immunization was O. D. = 0.146 to 0.347. All *T. aduncus* received a primary injection i.m. of $500 \mu\text{g}$ carbohydrate on 3 November 1987 and a booster injection of $500 \mu\text{g}$ car-

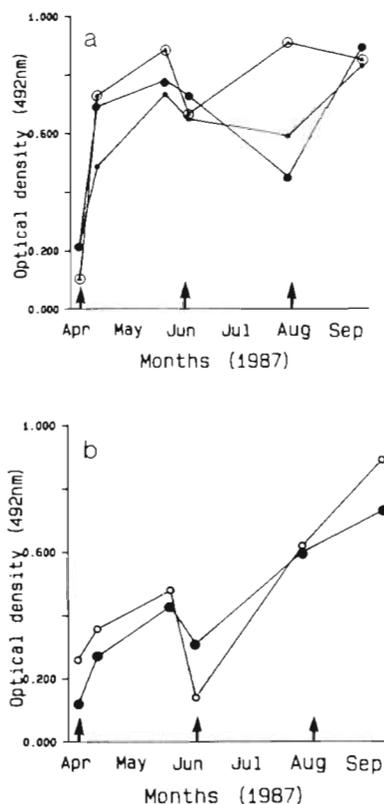


Fig. 1. Enzyme immunoassay (EIA) antibody levels in *Pseudorca crassidens*. (a) EIA values for 3 individuals (representative of a group of 7) through the rainy season (Apr–Dec) 1987. (b) Two individuals of the same group with lower antibody levels after immunization. Arrows: dates of immunization

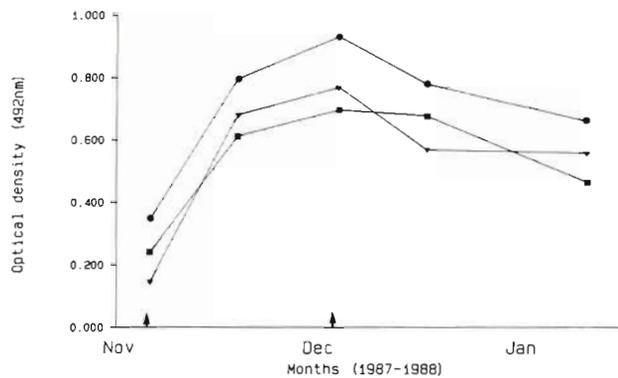


Fig. 2. Enzyme immunoassay antibody levels of 3 *Tursiops aduncus*, representative of a group of 8, during winter (Nov-Jan 1988). Arrows: dates of immunization

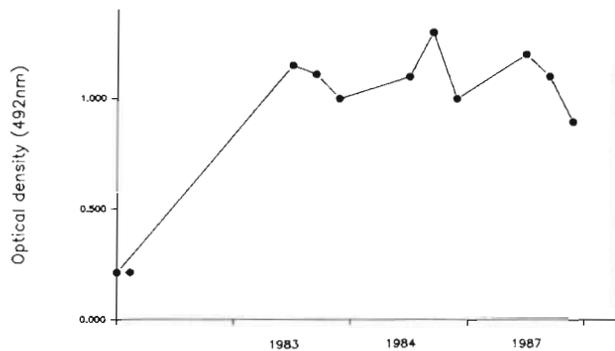


Fig. 3. Enzyme immunoassay antibody levels in 1 *Tursiops aduncus*, representative of 7 individuals, resident in Ocean Park 1983-1988. Initial antibody levels (arrow) is from the individual at capture in March 1982. Other antibody values were from bleedings in April, August and November of the years indicated. See 'Methods and Materials' for dates of immunization

bohydrate on 3 December 1987. The booster injection did not cause an increase in antibody levels.

The other resident dolphins (6 *Tursiops aduncus*, 1 *Lagenorchus obliquidens*) had essentially the same or higher EIA antibody levels during the 5 yr period of the vaccine trial as shown for the false killer whales in Fig. 1a (O. D. = 0.4 to 1.2). A representative example of 1 *T. aduncus* EIA antibody levels during 1983, 1984, and 1987 are shown in Fig. 3.

Four *Tursiops gilli* died from acute melioidosis during their first year of captivity (1986). Necropsis revealed pathology and microbiology consistent with diagnosis of melioidosis as previously reported (Liong et al. 1985). EIA values for 2 of these 4 individuals are shown in Fig. 4. Initial antibody levels to *Pseudomonas pseudomallei* a few days after capture, prior to immunization, and while the animals were still in the waters off the coast of Japan, were low. Antibody levels rose after initial immunization with 1 mg of the strain SLT *P.*

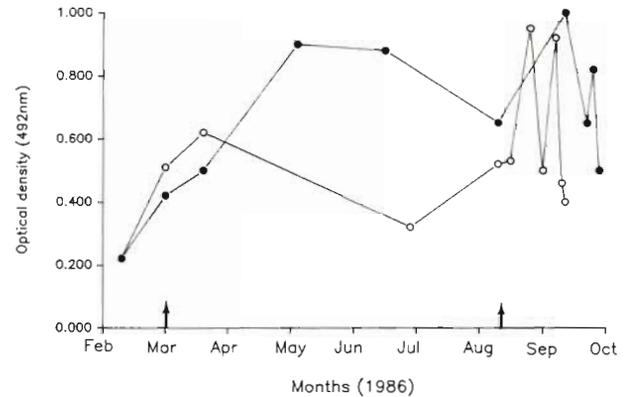


Fig. 4. Enzyme immunoassay antibody levels of 2 *Tursiops gilli* that died of melioidosis during their first year of captivity in 1986. Arrows: dates of immunization

pseudomallei vaccine (March) and were maintained during the rainy season with a single booster injection of 500 µg carbohydrate in mid-August. Antibody levels then dropped precipitously a few days later, rose the following day, then dropped at death 2 d later.

Immune response in a killer whale

A single resident killer whale *Orcinus orca* received an annual (Apr) injection of 1 mg carbohydrate (0.3 to 0.5 µg kg⁻¹) and boosters of 500 µg in July and September during a 5 yr period. At the start of the vaccine trial in 1983 the EIA antibody level was very high; (O. D. = 1.55) and this high range (O. D. 1.3 to 1.6) has been maintained to now.

DISCUSSION

Pseudomonads are ubiquitous in the environment and difficult to speciate (Redfern et al. 1966, Palleroni 1984). Biochemical/morphological criteria indicate that the *Pseudomonas* species most similar to *P. pseudomallei* are *P. mallei* (etiological agent of glanders), and *P. carophyllia* (a plant pathogen). Further, in one laboratory-acquired case of melioidosis, *P. pseudomallei* was mistaken for *P. cepacia*, a common bacterial inhabitant of humans (Schlech et al. 1981). In the present study there was no difficulty in isolating pure cultures of *P. pseudomallei* from the tissues of animals that died of melioidosis using crystal violet and gentamycin as selective inhibitors.

An extensive study is now in progress to determine the foci of *Pseudomonas pseudomallei* in the environment at Ocean Park. During the rainy season cultures of soil from multiple sites, fish preparation rooms, rainwater, etc. contained many saprophytic bacteria and fungi which readily grew on the selective medium

being used. Other selective inhibitors are being evaluated to facilitate this study.

There is no available vaccine for melioidosis either in humans or other animals. Extensive serosurveys of humans in SE Asia indicate very high rates of subclinical infections (Nigg 1963) but rare acute infections. *Pseudomonas pseudomallei* is not a very invasive pathogen (Redfern et al. 1966) but can readily cause disease whether in war wounds (Vietnam) (Weber et al. 1969) or when water is aspirated during drowning (Achana et al. 1985, Lee et al. 1985).

Only recently has it been recognized that, in Asia, captive animals such as gorillas, deer, and particularly cetaceans are susceptible to melioidosis during specific times of the year (Vedros et al. 1983). Prior to 1983 a sonicated lysate preparation of several strains of *Pseudomonas pseudomallei* was first tested as a vaccine. The strains included those from the elephant seal, and 6 from *Tursiops aduncus* including strain SLT. The lysate was effective in protecting hamsters experimentally and produced high levels of complement fixing antibodies in pinnipeds and cetaceans but was very toxic for cetaceans. This toxicity, as observed by the authors and animals trainers, consisted of reduced food intake, lethargy, erratic behavior and refusal to participate in any behaviour patterns for several days. The sonicated lysate vaccine preparation caused no side reactions in pinnipeds.

This report describes a vaccine which consists of a protein-polysaccharide mixture (1:3), as yet not fully defined chemically, which produced no side reactions in cetaceans, was effective in protecting hamsters experimentally, induced high levels of specific antibodies, and has reduced mortality in cetaceans due to *Pseudomonas pseudomallei* from 45 % in 1983 (D. D. Hammond pers. comm.) to less than 1 % in 1988.

We have no explanation for the death in 1986 of the 4 *Tursiops gilli* with high levels of antibodies (Fig. 4) nor for the erratic antibody behaviour prior to death. It may be that these dolphins had foci of internal chronic lesions consistently noted at necropsy in animals that died of melioidosis (Liong et al. 1985). *Pseudomonas pseudomallei* could then be released into the blood stream. Another possibility is that these dolphins were subjected to a massive challenge of *P. pseudomallei* in the water, and/or possibly compromised by severe stress. The current speculation is that *P. pseudomallei* is an opportunist, concentrated in the cetacean environment during the rainy season in Ocean Park, and may play a role in the terminal illness of animals compromised by other infections. In 1 *T. aduncus* that died in 1988 there was evidence of viral hepatitis by histology, yet *P. pseudomallei* was isolated from many tissues

(Jay Sweeney pers. comm.). Studies on environmental sources of *P. pseudomallei*, appropriate dosages of vaccine, and optimum time intervals of immunization are in progress.

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