CHAPTER II

MATERIALS AND METHODS

1. Experimental Animals

1.1 Mice

Male BALB/cJ mice 8 to 12 weeks of age were kindly supplied by Department of Veterinary Medicine, AFRIM, Bangkok and used througout these studies. Five mice were kept in each cage, and the mice were given unlimited supplied of food and water in an isolated air conditioned.

1.2 Rabbits

New Zealand white rabbits were kindly supplied by Science Division, Thai Red Cross Society (Queen Saovabha Memorial Institute), Bangkok.

2. Bacterial Strains

2.1 Description of Strains

These are summarized in Table 2. All these strains were kindly supplied by Department of Microbiology and

Table 2 Description of bacterial strains

Strain	Description	Serotype	
		O Ag	H Ag
S.typhimurium F885	his+ hybrid from *E. coli* Hfr59x*S.typhimurium* 1591 (his-)	*E.coli* 8	i:1,2
E.coli F492	K-mutant of *E.coli* E 56b (O8:K27)	8	non motile
S.typhimurium C5		1,4,5,12	i:1,2
S.strasbourg		9,46	d:1,7
S.enteritidis 11RX		1,9,12	g:m
S.typhimurium M206		1,4,5,12	i:1,2

Immunology University of Adelaide, Australia.

The hybrid strains, F885 were originally received from Dr. G.Schmidt of the Max Planck Institute, Freiburg, Federal Republic of Germany.

2.2 Strain Maintenance

All these strains were stored as lyophylized culture at 4°C. For routine use, all lyophylized cultures were grown on tetrazolium-lactose (TZ/LAC) agar plates (see Appendix I-3) and a single colony was transferred and kept in semi-solid agar (see Appendix I-2) stock bottles at 4 C.

2.3 Strain Propagation

Culture transferred from semi-solid agar stock bottles were grown on TZ/LAC agar plates and a single colony was transferred to brain heart infusion (BHI) broth at 37°C for 18 h. These cultures were diluted 1:10 with fresh BHI broth and incubated as a shaking culture for 3 h. This method yielded organisms that were in the log arithmic growth phase. The number of bacteria present was calibrated by optical density at wavelength of 650 nm and confirmed by viable counts. The ultimate number of bacteria in such cultures was about $2x10^9$ organism/ml

3. Heamagglutination Test

Acid citrate dextrose-treated GPE (see Appendix II-1.2) were washed twice in NSS and made up to 1% in 0.1 M PBS-BSA (see Appendix II-1.4) 50 µl of a 1% GPE was added to an equal volume of the test bacterial suspension or fimbrial suspension and the contents were mixed and left overnight at 4°C before reading

3.1 The minimum haemagglutinating dose (MHD) of bacteria from a culture was measured as the smallest number of bacteria/ml in the mixture with red cells that gave visible HA.

3.2 The minimum haemagglutinating concentration (MHC) of the fimbrial suspension was measured as the smallest concentration of protein/ml in the mixture with red cells that gave visible HA.

3.3 The haemagglutinating power (HP) of a culture was measured as 10^{11} divided by MHD of bacteria/ml (12).

4. Haemagglutination-Inhibition Test

Serial doubling dilution of a 10% solution of the carbohydrate were made with a 1% suspension of GPE as diluent (see Appendix II-1.3). 50 µl of each dilution was mixed with equal volume of the fimbriate bacterial

suspension (4MHD) and left overnight at 4°C before reading.

4.1 The minimum inhibitory concentration (MIC) of the carbohydrate was the smallest concentration of carbohydrate in the mixture with bacteria and red cells that completely prevented HA.

5. Fimbrial Preparation

After growth for 48 h in static, aerobic BHI broth, the *S. typhimurium* F885 fimbriae were purified according to:

5.1 The method of Dodd and Eisenstein (101)

The bacteria were collected by centrifuging for 10 min at 10,000 xg at 4°C. Bacteria were washed with 0.5% NaCl and resuspended in 5 mM Tris buffer, pH 7.8 (see Appendix II-2.1.1). Fimbriae were removed by mechanical agitation at high speed in a Ultra-Turrax homogenizer using two times for 1 min bursts. The defimbriated cells were sedimented at 27,000 xg for 30 min, and the supernatant was then subjected to ultra centrifugation at 270,000 xg for 2 h. The pellet of semi-pure fimbriae was resuspended in a small volume of 5 M urea 5 mM tris, pH 7 (see Appendix II-2.1.2), and incubated for 18 h at 37°C. The urea buffer mixture was subsequently diluted to 1 M by addition of an appropiate volume of Tris buffer. This suspension was layered on top of an equal volume of 1 M sucrose-1 M urea-

5 mM Tris (see Appendix II-2.1.3), and pure fimbriae were obtained by centrifuging for 16 h at 200,000 xg in a 55P-72 Ultra-centrifuge (HITACHI Instruments).

5.2 <u>The method of Knutton et al.</u> (102)

Bacteria were havested and suspended in 0.1 M PBS, pH 7.2 (see Appendix II-2.2.1) and sheared by three 5-min treatments in a Ultra-Turrax homogenizer (Janke and Hunkel, West Germany) with cooling between each shearing. The sheared homogenate was clarified by centrifugation at 8,000 xg for 15 min, and outer membrane vesicles were removed by centrifugation for 2 h at 45,000 xg. The supernatant was removed and centrifuged at 190,000 xg for 2 h, and the pellet containing fimbriae was suspended in a small volume of PBS. The fimbrial suspension was applied to a self-generating isopycnic cesium chloride gradient with a density of 1.29 g/cm^3 (see Appendix II - 2.2.2) and centrifuged at 160,000 x g for 18 h.

5.3 <u>The method of Salit and Gotschlich</u> (62)

The bacteria were collected by centrifugation at 10,000 xg at 4 C for 20 min in a J2-21 centrifuge (Beckman Instruments) and resuspended in 0.05 M Tris-HCl, pH 7.8 (see Appendix II-2.3.1). This bacterial suspensions was then mixed at top speed for 2 min in a Sorvall Omnimixer (Dupont Instruments). The defimbriated bacteria were

sedimented at 10,000 xg at 4°C for 20 min. The supernatant was decanted and spun at 10,000 xg for 30 min to remove any remaining bacteria. This solution was then dialysed against 0.1 M sodium acetate buffer, pH 3.9 (see Appendix II-2.3.2) and the aggregated fimbriae removed by centrifugation at 2,000 xg for 20 min. The sediment was washed in sodium acetate buffer and the pellet resuspended in Tris buffer. Saturated ammonium sulfate (see Appendix II-2.3.3) was filtered and added dropwise to this stirred solution to a final volume of 10%. Fimbrial aggregates were then collected by centrifugation at 4,000 xg for 15 min and resuspended in Tris buffer. The precipitation procedure was repeated twice more. Fimbriae were mixed with cesium chloride so that the density of the mixture was 1.30 g/cm^3 (see Appendix II-2.3.4) and the fimbrial concentration 100 µg/ml. Centrifugation was then carried out using polyallomer tubes at 4°C with a swinging bucket rotor at 160,000 xg for 18 h in a L8-70 Ultra-centrifuge (Beckman Instruments).

6. <u>Protein Estimation</u>

Protein content was estimated according to Lowry et al. (103), with bovine serum albumin as the standard. To 0.5 ml of sample was added 3 ml of solution C (see Appendix II-3.3). This mixture was blended with a Vortex mixer and allowed to react for 10 min at room temperature, 0.3 ml of solution D (see Appendix II-3.4) was added and the solution

was immediately mixed. The samples were incubated for 30 min at room temperature and then read in a spectrophotometer at 650 nm.

7. Electron Microscopy

The samples were applied to copper grids coated with colloidion and carbon. Samples were negatively stained with 2% (w/v) of phosphotongstic acid in 0.1 M PBS, pH 6.5 (see Appendix II-4.1) and examined with an JEM-200 CX electron microscope (JEOL Instruments).

8. Determination of Fimbrial Purity and MW

8.1 Immunoelectrophoresis

IEP was performed with 1.5% noble agar (see Appendix II-5.2) in sodium barbital buffer, pH 8.2 (see Appendix II-5.1) at a voltage of 150 Vott, 1 1/2 h. Depending upon the experiment, the troughs were filled with rabbit serum prepared against either the whole cell of *S. typhimurium* F885 Ag or purified type-1 fimbriae Ag.

8.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed in 0.6-mm thick, 12.5% slab gels by the system of Laemmli (104).

8.2.1 Preparation of Electrophoresis Gels

The separating gel mixture (see Appendix II -6.2) was poured in the glass plate. Taking care that no air bubbles were trapped in the mould. Before the gel hardens, a few drops of water were carefully layered on top of gel solution. After polymerization was complete, the water was decanted from top of gel and the stacking gel solution (see Appendix II-6.3) was added. The gel was kept at room temperature for about an hour to allow complete polymerizaton.

8.2.2 Electrophoresis

Samples of fimbriae were dissociated before electrophoresis by being heated in acid (18,105) to 100 °C for 5 min, cooled, neutralized, mixed 4:1 with sample buffer (see Appendix II-6.4) heated to 100 °C for 2 min, and applied to the gel. The compartments of electrophoresis apparatus were filled with electrode buffer (see Appendix-II-6.5). A constant current of 15 mA was then applied until the marker dye had moved to 1 cm from the anodal end of the gel.

8.2.3 Staining and Destaining

The gel was immersed in staining soluton (see Appendix II-6.8) for at least 1 h, then destained

by diffusion against several changes of fixing solution (see Appendix II-6.7). The gel was stored in the destaining solution (see Appendix II-6.9).

8.2.4 Estimate of the MW

To determine the relative mobility (Rf) of a protein, its migration distance from the top of gel to the center of the protein band was divided by the migration distance of the bromphenol blue tracking dye from top of the gel. The Rf values were plotted against the known molecular weights on semi-logarithmic paper (Fig.3).

9. Immune Serum

9.1 Ab Against S.typhimurium F885 and E.coli F492

Rabbits were immunized by subcutaneous injections at 4 sites of 5×10^8 heat-killed bacterial cells suspended in NSS, emulsified with an equal volume of Freund's complete adjuvant, every week for 4 weeks and the rabbits were bled 7 days thereafter.

9.2 Ab Against Type-1 Fimbriae

500 µg purified type-1 fimbriae were suspended in 1 ml emulsified with an equal volume of Freund's complete adjuvant, and injected subcutaneously into rabbits every

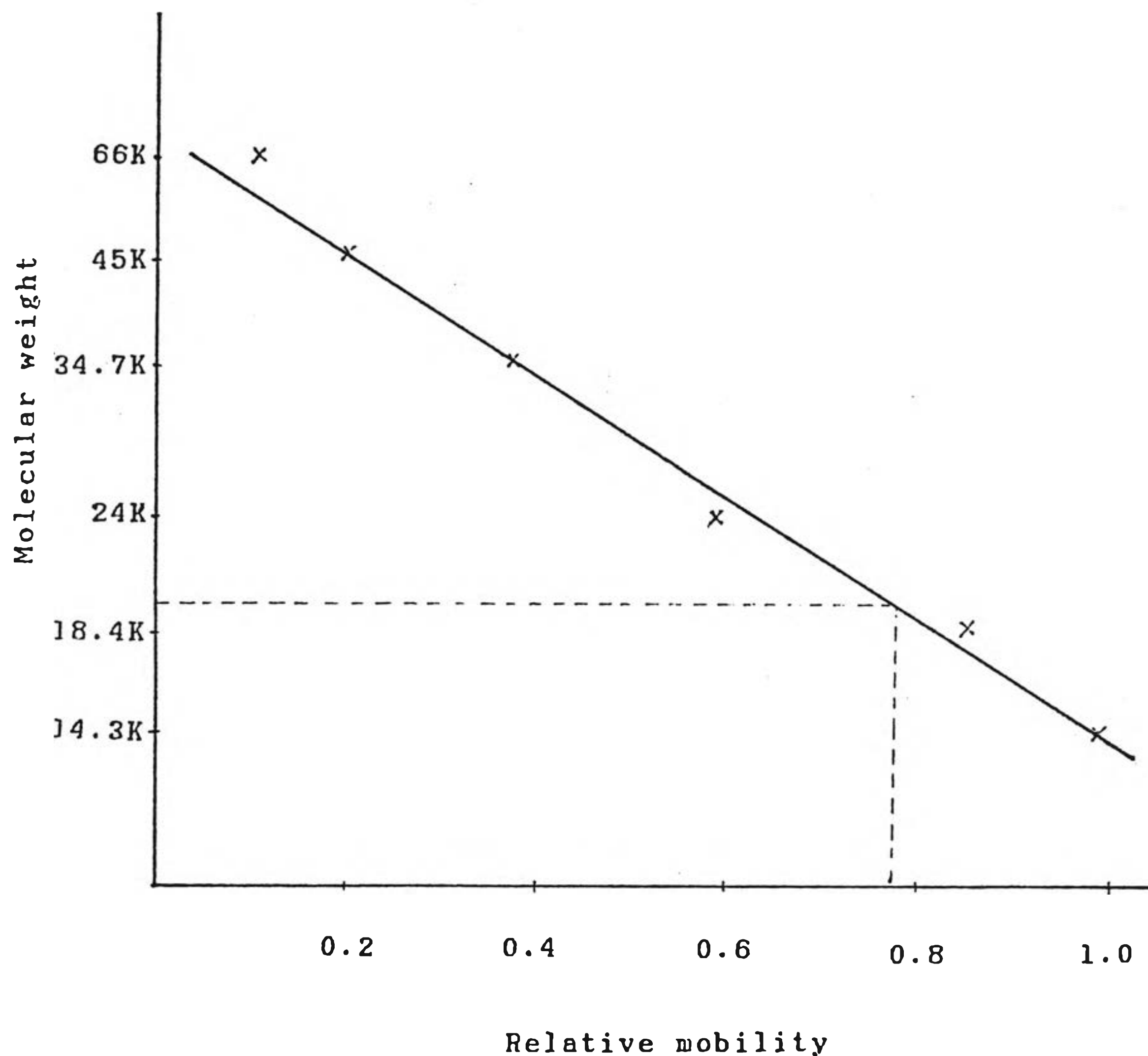

Fig.3 Calibration curve for MW determination by SDS-PAGE. Standard protein were BSA (66K), ovalbumin (45K), pepsin (34.7K), trypsinogen (24K), B-lacto globulin (18.4K), and lysozyme (14.3K).

week for 3 weeks; they were bled 7 days after the last of injection.

Blood was obtained by puncture of the ear vein and was clotted at room temperature, the serum was collected and the presence of Ab against whole bacteria and purified fimbriae were checked by a bacterial agglutination test.

Salmonella O group B antiserum (BBL) was used for Ab against S.typhimurium C5

10. Bacterial Agglutination

Slide agglutination were performed by placing 20 µl of antiserum and a loop of bacterial colony on a microscope slide. Agglutination was assessed visually over a 1-min period compared to a control suspension of the bacteria in NSS.

11. LD50 Determination

Groups of eight mice were fed orally with ten-fold dilutions of S.typhimurium C5. Mortality was recorded over 30 days, and the LD50 determined by the method of Reed and Muench (106).

Proportional distance

$$= \frac{50\% - (\text{mortality at dilution next below})}{(\text{mortality next above}) - (\text{mortality next below})}$$

LD50 = [log of dilution titer lower than 50% mortality + (proportional distance x log of dilution factor)]

12. Oral Immunization and Oral Infection

Mice were fed 0.2 ml of 50% saturated $NaHCO_3$ solution (see Appendix II-7.1) followed in a few minutes by 0.2 ml of a suspension of the immunizing or challenge organism. These were delivered intragastrically by a syringe fitted with a blunt-ended 19 gauge needle.

13. Recovery and Enumeration of Bacteria from Mice

At specified times after oral dosing, groups of five mice were killed by cervical dislocation, and their spleens and Peyer's patches were removed aseptically. Organs were homogenized separately in ice-cold saline with an Ultra - Turrax homogenizer. These homogenates were appropriately diluted and spread on the surface of TZ/LAC agar, which were then incubated for 18 h at 37°C. The recovered colonies were counted, and the number of organisms recovered from the homogenized Peyer' patches and spleen from each mouse was recorded and the group average

obtained. The identity of the bacterial colonies was confimed by agglutination in specific antiserum.

14. Mouse Protection Test

14.1 Immunization

Group of mice (30-40 per group) were immunized with either the $1x10^{10}$ viable organisms of *S.typhimurium* F885 or *E.coli* F492 by oral route. The third group was immunized with 50 µg of fimbriae intraperitonealy on day 0 and subcutaneously on day 12. The control for each group received saline.

14.2 Determination of Protection

Control and immunized groups were challenged with a lethal doses of 1,000 LD50 of *S.typhimurium* C5 (mouse typhoid) orally 21 days after bacterial immunization and 15 days after fimbrial immunization.

14.2.1 The daily death count was recorded, and after 30 days the percentage of survival in each group was calculated.

14.2.2 Enumeration of viable *S.typhimurium* C5 in the Peyer's patches and spleen of both control and immunized groups.