**Annex 1:** Fowl cholera Inoculum and production media

| No | Chemical type | Unit | Quantity in liter | Remark |
|---|---|---|---|---|
| **1** | **Peptone** | **Gram** | **10** | |
| 2 | Casitonehydrolysate | **Gram** | 5 | |
| 3 | Sodium chloride | **Gram** | 5 | |
| 4 | Di sodium hydrogen sulphate (Na2HPo4 | **Gram** | 7 | |
| 5 | Potassium Di hydrogen sulphate (KH2Po4) | **Gram** | 2.5 | |
| 6 | Magnesium sulphate (MgSo47H2O) | **Gram** | 1 | |
| 7 | Yeast extract | **Gram** | 2.5 | |
| 8 | D (+) glucose | **Gram** | 5 | |
| 9 | Horse serum | ml | 10 | 10% |
| 10 | Distilled water | ml | 990 | |

Source: OIE, 2000

**Annex2:** Phosphate buffered solution (PBS)composition and preparation

| Salt | Concentration (mmol/L) | Concentration (g/L) |
|---|---|---|
| NaCl | 137 | 8.0 |
| KCl | 2.7 | 0.2 |
| Na2HPO4 | 10 | 1.42 |
| KH2PO4 | 1.8 | 0.24 |

1. The preparation was started with 800 mL of distilled water to dissolve all salts.

2. Distilled water was added to a total volume of 1 liter.

3. The resultant 1× PBS was with a final concentration of 157 mM $Na^+$, 140mM $Cl^-$, 4.45mM $K^+$, 10.1 mM $HPO_4^{2-}$, 1.76 mM $H_2PO_4^-$ and a pH of 7.96.

4. To shift the buffer, 2.84mM of HCl was added to 7.3 mM $HPO_4^{2-}$ and 4.6 mM $H_2PO_4^-$ for a final pH of 7.4 and a $Cl^-$ concentration of 142 mM.

The pH of PBS is ~7.4.

**Annex 3:** Tryptic soy broth (Oxoid Ltd., Hampshire, England) composition and preparation.

| Composition of Tryptic Soy broth | per liter of deionized water |
|---|---|
| Pancreatic Digest of Casein | 17.0gm |
| Peptic Digest of Soybean Meal | 3.0gm |
| Sodium Chloride | 5.0gm |
| Dipotassium Phosphate | 2.5gm |
| Dextrose | 2.5gm |
| Final pH = 7.3 +/- 0.2 at 25ºC. | |

1. The medium with 30 grams was suspended in one liter of distilled water.
2. It was mixed well and dissolved by heating with frequent agitation.
3. It was boiled for one minute until complete dissolution.
4. It was dispensed into appropriate containers and sterilized in an autoclave at 121 ºC for 15 minutes.
5. Larger quantities were required a longer sterilization time, but the temperature was not increased.

**Annex 4:** Composition and preparation of Skim Milk Powder

| Analysis | |
|---|---|
| Water | <5.0% |
| Ash | <10% |
| Total nitrogen | ~5.3% |
| Lipid content | < 1.5% |
| Reducing sugars(as lactose monohydrate) | ~55% |

Reconstitution:

The powder was mixed to a smooth paste with a small quantity of distilled water, and then gradually more distilled water was added until a 10% w/v mixture was obtained. This was equivalent to fresh milk, and sterilized by autoclaving for 5 minutes at 120℃. Special care was taken not to overheat during sterilization to avoid occurrence of caramelization.

**Annex 5:** Estimation of colony forming unit using plate count method

1. At a hemolytic tube 4.5 ml 7 hours incubated inoculated *P. multocida*biotype A culture was prepared.
2. From this culture 0.5 ml was added to 4.5 ml of peptone water.
3. From this composition 0.1 ml was transferred to 4.5 ml of peptone water.
4. Then it was homogenized well and transferred 0.5 ml to another hemolytic tube up to $10^{th}$ tube with peptone water.
5. Finally transferred to TSA plate.
6. A number of two TSA plate was prepared for one tube with peptone water.
7. For 10 peptone water tubes, 20 TSA plates were prepared and 0.1 ml was streaked in one plate and 0.1 ml in the second plate and incubated at 37℃ overnight.
8. Then the colony was counted manually.

9. The culture was harvested at the pH of 5.5 to 6.2, which is known to correspond to the desired titer.

**Annex 6:** Tryptone Soya Agar (Difco, spark, USA) composition and preparation.

| Ingredients | Grams/Liter |
|---|---|
| Casein peptone (pancreatic) | 15.0 |
| Soya peptone (papainic) | 5.0 |
| Sodium chloride | 5.0 |
| Agar | 15.0 |
| Final pH 7.3 +/- 0.2 at 25°C | |

Prepared media was stored below 8°C, protected from direct light. The dehydrated powder was stored, in a dry place, in tightly-sealed containers at 2-25°C.

**Directions**: Dehydrated media about 40g was suspended in 1 liter of purified filtered water. It was sterilized at 121°C for 15 minutes and cooled to 45- 50°C. It was mixed gently and dispensed into sterile Petridishes or sterile culture tubes.

**Annex 7:** Procedure of Gram Staining

1. A clean, grease free heat fixed slide was taken and placed on the staining rack.
2. The smear of suspension on the clean slide with a loopful of sample was prepared
3. Air dried and heat fixed
4. Crystal Violet staining solution was poured and kept for about 1-2 minutes and rinse with distilled water.
5. The gram's iodine was flooded over the smear for 1-2 minutes and washed with distilled water.
6. Then, the smear was washed with 95% alcohol for about 15-20 seconds and rinsed with water.

7. Safranin was added over the smear and allowed it to stand for about 30 sec-1 minute and washed with distilled water.
8. Air and blot dried the smear with blotting paper and Observed under Microscope (100*oil immersion objective).

**Annex 8:** Operational Instruction for vortex mixer(QMS-Sero-VOR-14)

1. The vortex mixer was pluged in the main power supply.

2. It was switched on the power button.

3.The test tube was firmily secured in place.

4. The speed knob was rotated to clock wise direction to adjust the speed.

5.The start or stop button was pressed to start.

6. The start or stop button was pressed when the work was stopped

7. The power button was switched off.

8. The power supply was disconnected.