3. MATERIAL AND METHODS

3.1 Study design

The study was performed as a cross sectional and a prospective study. Samples were collected during January – February 2007.

3.2 Sample size determination

Prevalence of *Salmonella* in breeder sows has never been reported before in Thailand. Therefore, in order to estimate the prevalence of *Salmonella* infection in breeder sows in Chiang Mai province, the prevalence data at fattening farm level was used (62.9 %, Dorn-in, 2005) with a maximum allowable error of 8% and 95% confidence level, it was concluded that 20 breeder sows would serve the purpose estimate the prevalence.

3.3 Study population

One breeder farm was selected by random from a private company that supplied weaned pigs to fattening farms in Chiang Mai, Thailand. The farm had 5,000 sows separated in to two housing system, open and close with the same management.

Each house with the average group size of 30-40 sows was managed in a 2-week system. Seven days prior delivery the sows were moved to the cleaned and disinfected farrowing units. In the farrowing unit sows were treated with antibiotic in order to prevent diseases. The piglets were weaned at 18-21 days of age and the sows were moved to the mating unit the same day. The all in all out system in mating, farrowing and nursery unit were used.

3.4 Sample selection

3.4.1. Animal samples

A total of 20 breeder sows was randomly selected ten sows from open house and other ten sows from close house. Simultaneously the sow ID was recorded. Three types of samples were collected from individual sows, blood sample, feacal samples and skin swab samples. Blood samples were collected at day 1 after delivery. Both fecal and skin swab samples were collected at day 1, 7 and 18 after delivery.

3.4.2. Environmental samples

Three types of environmental samples were collected from both housing systems at each visitation; water, floor swab and feed samples. Water included (i) water used for cleaning and disinfection and (ii) water for drinking. Floor swabs were taken from selected pens and feed was taken from the feed container (about 25 grams). The total of samples is shown in Table 8.

Table 8: Total samples taken

Day after delivery	Type of samples	Open house	Close house	Total samples
1	Blood	10	10	66
	Faecal	10	10	
	Skin swab	10	10	
	Environment	3	3	
7	Faecal	10	10	46
	Skin swab	10	10	
	Environment	3	3	
18	Faecal	10	10	46
	Skin swab	10	10	
	Environment	3	3	
Total		79	79	158

3.5. Sample collection

3.5.1. Serum samples

Blood (10 ml) of sows was taken at farm during farrowing status day 1 after delivery, into test tubes individually. Each tube was labeled with pig's unique identification number and was allowed to stand at room temperature for the blood to clot. After that serum was removed from each blood sample by (4000 rpm) centrifugation and stored at -20 ^{o}C until tested.

3.5.2. Faecal samples

Individual feces samples (25-30 g) from the selected sows were taken from the rectum, using a new disposal rubber glove. The fecal samples were submitted to the laboratory for examination within 3-4 hours of collection and examination was undertaken on the same day of receipt.

3.5.3. Skin swab samples

Individual skin swabs (100 cm^2) from the sows were kept in Buffered peptone water. The tube was sealed and sent to the laboratory within 3-4 hours for analysis.

3.5.4. Pen Swab Samples

At the day of fecal samples collecting, the pens were tested for *Salmonella* simultaneously. One sterile pair of gauze socks was used (socks consist of an elastic cotton tube pulled over the investigator's boots; each sock is approximately 20 centimeter long) to walk through the pens, the cotton tubes absorbing fecal material. The investigator turned the socks when walking (approximately 30 steps). So, all parts are exposed to the pen floor. After that, the soiled pair of sock was placed in a sterile plastic bag. Date, pen/house and farm was labeled, the sock were kept at 4 ^{o}C and tested with in 24 hours.

3.5.5 Water sample

Water samples filled in a sterile bottle. Samples were taken 1,000 ml and kept at 4 ^{o}C and send to the laboratory for testing within 24 hours.

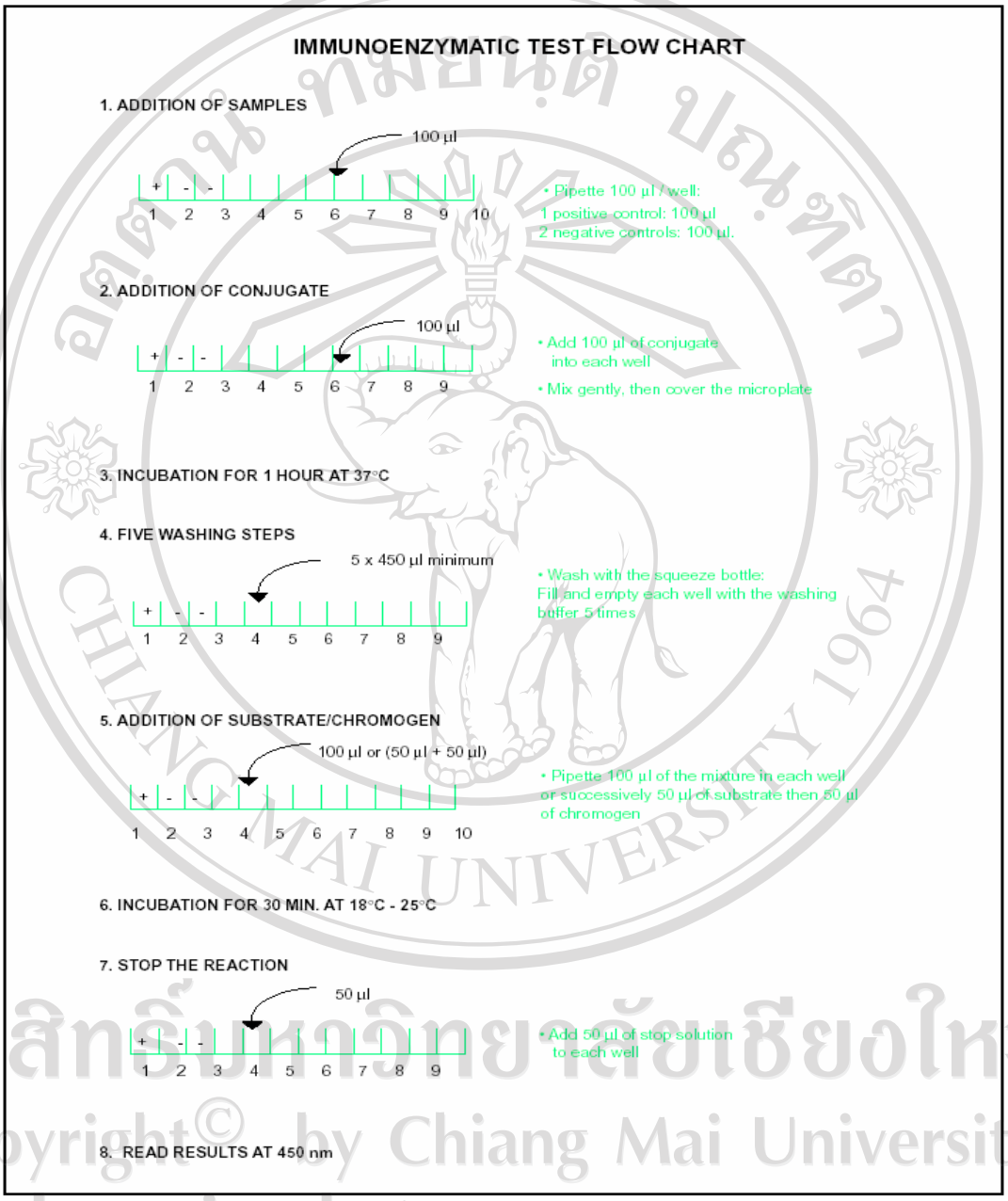
3.6. Laboratory Procedures

3.6.1. Serology: ELISA

The sera were tested for antibodies to *Salmonella* by the commercial test kits Danish mix- ELISA® (Labor Diagnostik Leipzig, Geramany). The kit is an enzyme immunoassay containing the O-antigens 1, 4, 5, 6, 7 and 12 isolates and measuring an optical density (OD) as a percentage of a known positive control. The Danish mix- ELISA® usually used to detect of antibodies to *Salmonella* in pork meat juice or pig serum, it detects more than 90% of the most common *Salmonella* serotypes.

Description and principles of ELISA

This assay is designed to measure the quantity of antibodies against *Salmonella* in pork meat juice or in pig serum. Salmonella antigen is coated on 96-well plates, upon incubation of the test sample in the coated well, antibodies specific to *Salmonella* form a complex with the coated *Salmonella* antigen. Unbound material is washed away and a conjugate is added which binds to any bound pork antibody in the wells. After washing away unbound conjugate from the wells, enzyme substrate is added. Subsequent color development from conjugate-bound enzyme is directly related to the amount of antibody to the *Salmonella* present in the test sample (Figure 3).



Source: Axelsson and Sorin (1997)

Figure 3: ELISA test flow chart

The ratio of the OD values of the Controls 1-5 and their indicated concentrations gives a linear regression straight line. The linear regression straight line is calculated by plotting the OD values of Control 1-5 on the X-axis versus the measured OD-values on the Y-axis. The antibody concentration of the samples has to be calculated by use of the straight-line formula. The concentration of the Control 1-5 is specified in the attachment to these instructions.

- Cut-Off values for samples (serum, meat juice, plasma);

  ≥ 40 OD% positive

  20 - < 40 OD% weak positive

  10 - < 20 OD% doubtful (positive)

  < 10 OD% negative

- Cut-Off values of Samples for categorization of stocks according to monitoring programs:

  ≥ 40 OD% or ≥ 20 OD% are positive depending on national regulations

For the assay to be valid, the P/N ratio between the Positive Control Serum 1 (P) and the Negative Control Serum (N) should be greater than 4.0.

3.6.2. Conventional culture methods

Conventional culture methods used were slightly modified from ISO 6579, generally four distinct phases or steps (Figure 4) can be describes:

First step (Non-selective pre-enrichment): Sample being blended in a nonselective medium, buffer peptone water and incubated at 37 °C for 18-24 hours to allow resuscitation of any stressed organism and growth of all organisms as well.

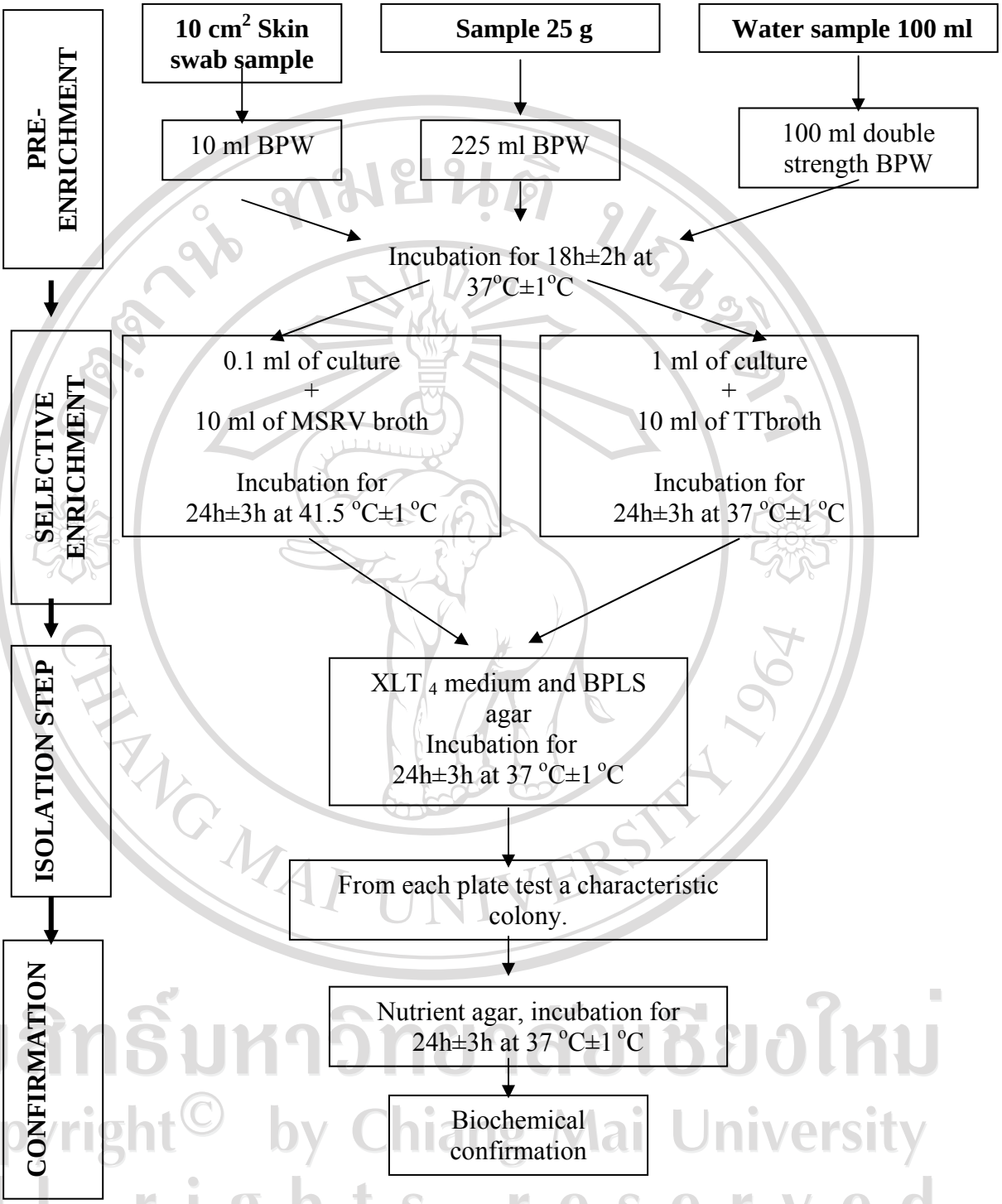
Second step (Selective enrichment step): Two types of selective enrichment media were used, first is Tetrathionate broth and another is Modified Semi-solid Rappaport-Vassiliadis broth (MSRV) after that incubation at 37 °C for 18-24 hours and 41.5 °C for 18-24 hours respectively.

Third step (Isolation step): in which selective enrichment media are streaked to selective solid agars containing one or more agents that inhibit non-salmonella organisms. XLT $_4$ and BPLS were used in this study.

Fourth step (Confirmation step): Characteristic colonies on the plates were submitted for biochemical test to confirm the isolate are member of the species *S. enterica*. Biochemical properties of Salmonella are shown in Table 9.

Table 9**:** Interpretation of biochemical test (WHO, 2001)

Medium	**Reactions/enzymes**	**Results**	
		Negative	**Positive**
TSI	Acid production from glucose	Butt red	Butt yellow
TSI	Acid production from lactose and/or sucrose	Surface red	Surface yellow
TSI	Gas production	No air bubble in butt	Air bubble in butt
TSI	Hydrogen sulfide production	No black colour	Black colour
Urea agar	Urease	Yellow	Rosa pink-deep cerise
MIL	Lysine decarboxylase	A yelow/brown colour	A purple colour and a yellow/brown colour in the AHD control medium
MIL	Motility	Bright purple colour	Turbidity
Indole	Indole production	Yellow ring	Red/pink ring



Source: Adapted from ISO 6579 (2002)

Figure 4**:** flow chart of *Salmonella*: Conventional methods

3.6.3. Serotyping

All isolates were serotyped by agglutination according to the Kauffmann-White scheme using *Salmonella* Polyvalent I, II (A-E, J-67) manufacture (Sifin, Germany) and *Salmonella* antiserum specific to individual group by the following process (Figure 5).

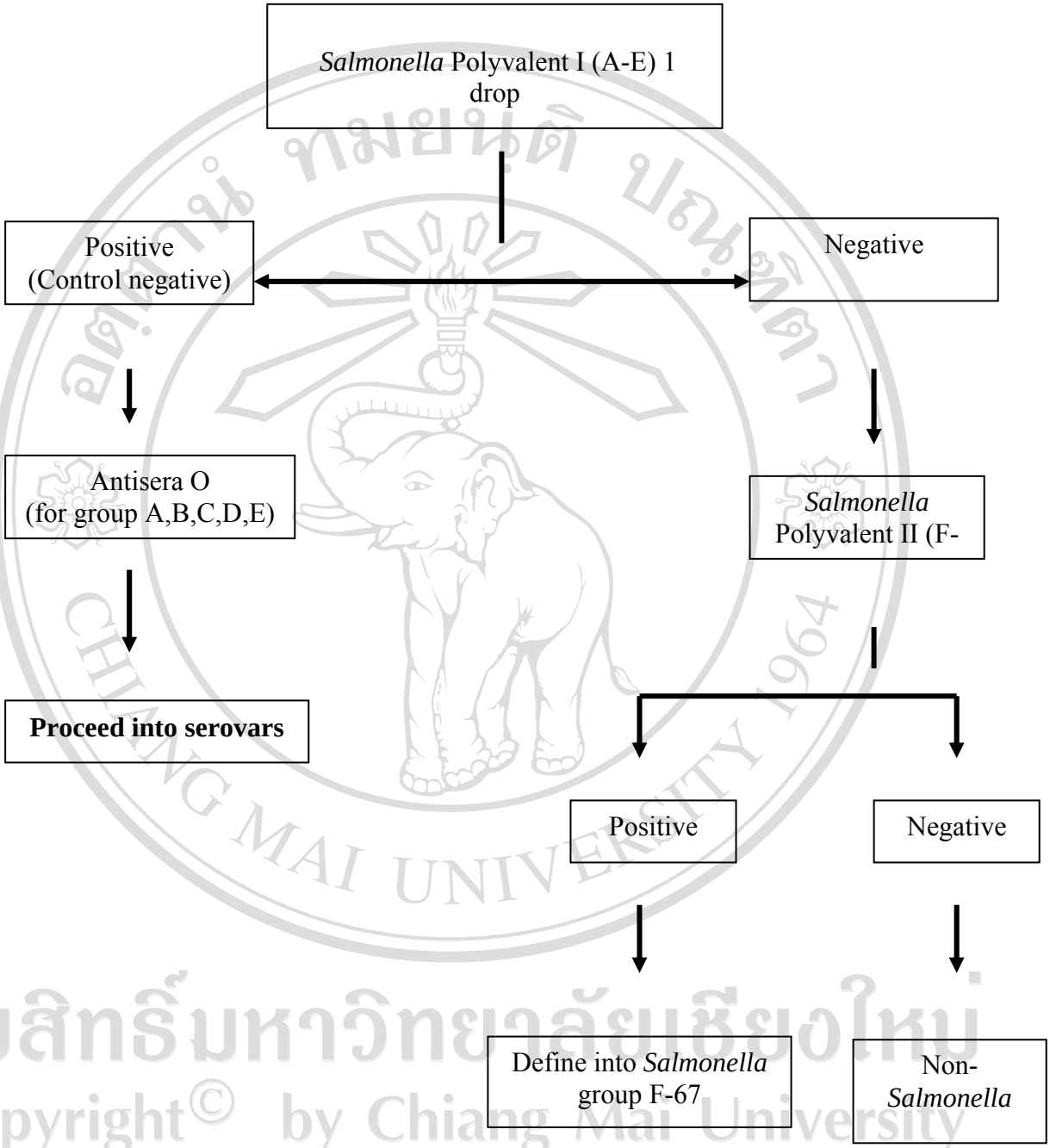
1. Test the selected colonies with *Salmonella* polyvalent I (A-E), if the agglutination is positive (+), the selected colonies posses the antigen to that antiserum, colonies will be regarded as a member of *Salmonella* Group A-E.

2. Test negative (-) result colonies (from the first step) with *Salmonella* polyvalent II (F-67), if the agglutination is positive (+), those colonies posses the antigen to that antiserum, colonies will be regarded as a member of *Salmonella* Group F-67.

3. *Salmonella* Group A-E will be processed by using the same type of colonies with specific antiserum of *Salmonella* group A, group B, group C, group D and group E by using a sequence of somatic antigen sera (Sifin, Germany). Sequence of testing based on information of the occurrence in Thailand and South East Asia.

4. Determination of flagella antigens, this step was done after transfer of the isolate to the motility agar. Performing agglutination for flagella antigen phase 1 and phase 2. If phase 2 did not appear, the serotype might be in the first phase only or vice versa. Then the challenge test was used where the antigens were to be blocked by the particular H antiserum to force the strain to develop the other phase (Sifin, Germany).

5. Diagnosis of the serotype of *Salmonella*.



The negative control used was NaCl solution

Figure 5**:** *Salmonella* serotyping flow chart

3.7 Data management and analysis

For descriptive analysis, Data from conventional *Salmonella* culture were entered into STATA V. 9 analyzed *Salmonella* prevalence in breeder sows and 95% confidence intervals (CI 95%). Distribution of *Salmonella* serotypes in animal and environmental samples from both housing system used the pivot table to add up the results .The incidence density of *Salmonella* during peripartureint period were calculated by this equation.

$$\frac{\text{New cases}}{\text{Population at risk x Time at risk}}$$

Kappa statistics were used for assessment of agreement between two different methods of *Salmonella* isolation, conventional culture of faecal samples and commercial ELISA test using Epi Info 2002. According to Dahoo *et al.* (2003), the criterion of kappa statistics was categorized into the following:

0.2- 0.4: fair agreement
0.4-0.6: moderate agreement
0.6-0.8: substantial agreement
>0.8: almost perfect agreement