CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Recruited dairy farms

According to the previous research in Germany, the herd prevalence of ESBL-producing *E. coli* was 30% (18/60) from tank milk samples (Kreausukon, 2011). Furthermore, an investigation in North West England and North Wales show that the overall prevalence of CTX-M *E. coli* positive cattle farms was 41.5% (27/65) (Snow et al., 2012). For logistic reasons, 5 districts (D1: Yanqing, D2: Daxing, D3: Miyun, D4: Tongzhou, D5: Fangshan) were selected and the assumed prevalence of ESBL- *E. coli* was 50%. There are 117 small farms (< 500 cattle), 37 large farms (equal or greater than 500 cattle) distributed in the 5 districts. With an accepted error of 10% and a level of confidence of 95%, the population adjusted sample size in small farms and large farms calculated by using Win Episcope 2.0 were 53 and 27, respectively. The sample size in each district was calculated as shown in the following two tables (Table 3 and Table 4).

Table 3. Sample size in different districts for small farms

Districts	D1	D2	D3	D4	D5	Total
Small farm (N)	38	11	41	11	16	117
Proportion	32%	9%	35%	9%	14%	100%
Samples (N)	17	5	19	5	7	53

Table 4. Sample size in different districts for large farms

Districts	D1	D2	D3	D4	D5	Total
Large farm (N)	7	10	7	7	6	37
Proportion	19%	27%	19%	19%	16%	100%
Samples (N)	5	8	5	5	4	27

3.1.2 Samplings at dairy farms

In each selected farm, five animals were conveniently selected and around ten grams fresh feces were collected from each animal using sterile plastic bags. The farm name and sampling time were marked on the bags. The samples were kept at less than 4°C and were protected against external contamination during storage and transportation. The samples were sent to the laboratory and reached the laboratory not later than 5 hours after sampling.

3.1.3 Questionnaire

Questionnaires (appendix) were used to investigate the management, disease controlling, amount and situations of using antimicrobials and other relevant information. Furthermore, the questionnaire included questions about milk quota, herd size and composition, heifer management, health status, mastitis prevent and treatment methods. The researchers asked questions and fill the questionnaires when they visited the farms and collected the samples.

3.2 Methods

3.2.1 Bacterial isolation

ESBL-*E. coli* were isolated in accordance with the directly plate method described by previous studies (Alexander et al., 2008, Kreausukon, 2011, Singer et al., 2008) and simply modified by using Maximum Recovery Diluent (MRD) to dilute the fecal samples. Briefly, two g per cow (n = 5) were pooled to a sample of 10 g and homogenized in 90 mL of MRD in sterile plastic bag. Aliquots of 0.01 mL were directly plated on MacConkey (MAC) agar plates and MacConkey containing 2 mg/L cefotaxime (MAC-CTX) plates (Figure 4). The streaked plates were incubated for 24 h at 37°C. Meanwhile, the homogenized samples in sterile bags were incubated for enrichment for 24 h at 37°C and aliquots were streaked on plates in case of negative results after the direct plating. If there were presumptive *E. coli* growed on the plates, colonies were streaked on Trypticase soy agar (TSA) to get pure cultures.

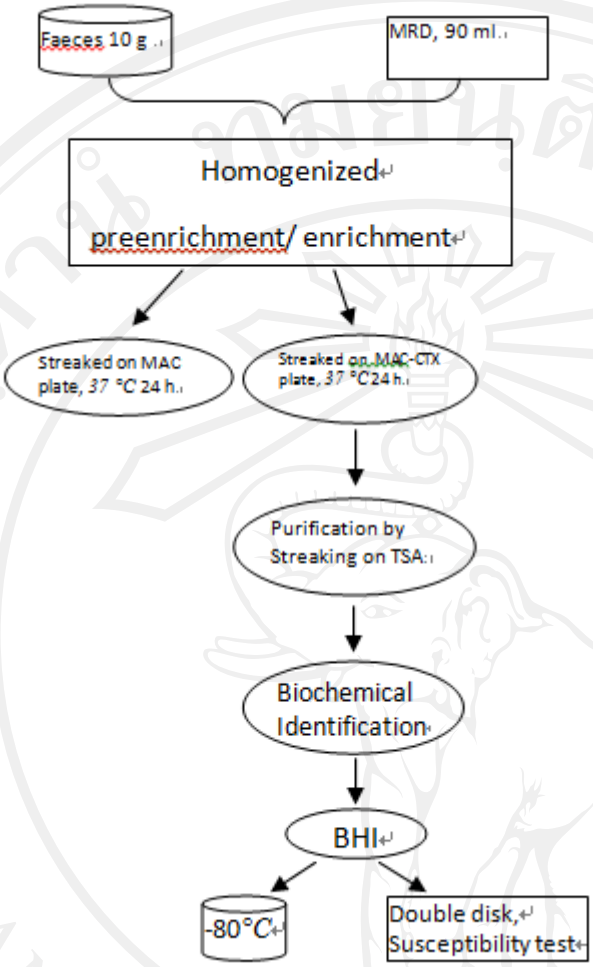

Figure 4. Flow chart of microbiological test procedure.

3.2.2 Identification of the presumptive *E. coli*

One colony from each suspected sample was confirmed as *E. coli* by using API 20 E kit (Biomerieux, Craponne, France) (Kreausukon, 2011). The isolate of confirmed ESBLs-producing *E. coli* were suspended in Brain Heart Infusion (BHI) broth and incubated at 37°C for 20 to 24 h. The suspension (0.9 ml) were added to 0.9 ml Luria-Bertani (LB) broth with 80% glycerol solution and the mixture were frozen at -80°C and -20°C for further identification and characterization (Kreausukon, 2011).

3.2.3 Screening and Confirmatory Tests for ESBLs

According to the Clinical and Laboratory Standard Institute (CLSI, 2012) recommendations, the disk diffusion method were used to screen and confirm the *Escherichia coli* (*E. coli*) as ESBL-producers. As described by Livermore and Brown (2001), bacteria were suspended equivalent to McFarland 0.5 turbidity standard in Mueller-Hinton broth. After inoculating a Mueller-Hinton Agar (MHA) plate, disks containing antimicrobials (Cefotaxime and Ceftazidime) as described in table 5 were used to carry out initial screen and confirmatory test (CLSI, 2012) (Figure 5). CLSI recommended performing phenotypic confirmation of potential ESBL-producing isolates of *E. coli* by testing both cefotaxime and ceftazidime, alone and in combination with clavulanic acid(CLSI, 2012). Testing was performed by disk diffusion and a $\geq$ 5 mm increased in a zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone confirms an ESBL-producing organism.

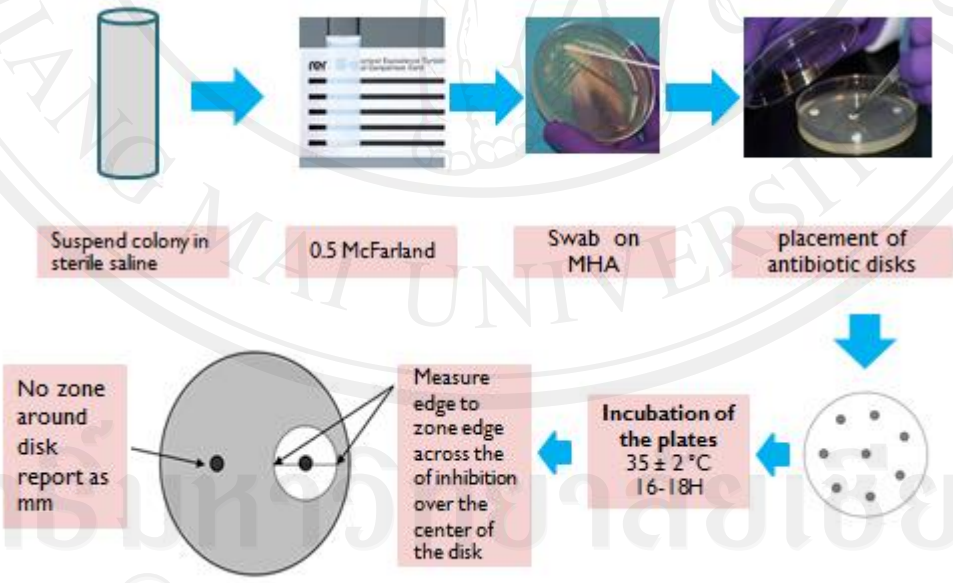

Figure 5. Flowchart of Screening / Confirmatory Tests for ESBLs and Antimicrobial susceptibility testing (CLSI, 2012).

Table 5. Screening and Confirmatory Tests for ESBLs in *Escherichia coli*

Test	Initial screen test	Phenotypic confirmatory test
Test method	Disk diffusion	Disk diffusion
Medium	MHA	MHA
Antimicrobial concentration	Ceftazidime 30 μg or Cefotaxime 30 μg	Ceftazidime 30 μg Ceftazidime-clavulanic acidb 30/10 μg and Cefotaxime 30 μg Cefotaxime-clavulanic acid 30/10 μg (Confirmatory testing requires use of both cefotaxime and ceftazidime, alone and in combination with clavulanic acid.)
Inoculum	Standard disk diffusion recommendations	Standard disk diffusion recommendations
Incubation conditions	35 ± 2 °C; ambient air	35 ± 2 °C; ambient air
Incubation length	16–18 hours	16–18 hours
Results	Ceftazidime zone ≤ 22 mm Cefotaxime zone ≤ 27 mm Zones above may indicate ESBL production.	A ≥ 5-mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanic acid vs its zone when tested alone = ESBL (eg, ceftazidime zone = 16; ceftazidimeclavulanic acid zone = 21).
Quality Control recommendations	*E. coli* ATCC 25922: Ceftazidime (25-32mm) Cefotaxime (29-35mm)	*E. coli* ATCC 25922: ≤ 2 mm increase in zone diameter for antimicrobial agent tested alone vs its zone when tested in combination with clavulanic acid.

3.2.4 Antimicrobial susceptibility testing

For ESBLs-producing *E. coli* isolates, antimicrobial susceptibility tests were performed using agar disk diffusion method as recommended by the Clinical and Laboratory Standard Institute (CLSI, 2012). For this purpose, Mueller Hinton agar (MHA) was used and colonies were harvested from the surface of the medium with a cotton swab after 24 h growth at 37°C.

The confirmed isolated colonies were suspended in sterile saline solution (0.85% NaCl) and the density was adjusted to a 0.5 McFarland turbidity standard. A sterile swab was used to dip into the sustention and the swab was evenly streaked on the MH agar plate. Antimicrobial-impregnated disks were placed on the surface of the agar plate. Once all disks were in place, and placed them in a 35°C air incubator for 16 to 18 hours.

Evaluation of antimicrobial resistance were based on performance standards for antimicrobial susceptibility testing as recommended by the Clinical and Laboratory Standard Institute (CLSI, 2012). For each drug the zone size was evaluated as susceptible (S), intermediate (I), or resistant (R) based on the interpretation chart of CLSI.

The tested antimicrobial agents as well as the interpretation of the results are shown in Table 6.

Table 6. Zone Diameter Interpretive Standards for *Enterobacteriaceae* (CLSI, 2012).

Antimicrobial agent	Disk content	Zone diameter breakpoints, nearest whole mm		
		S	I	R
Ampicillin	10 μg	≥ 17	14–16	≤ 13
Amoxicillin-clavulanic acid	20/10 μg	≥ 18	14–17	≤ 13
Gentamicin	10 μg	≥ 15	13–14	≤ 12
Kanamycin	30 μg	≥ 18	14–17	≤ 13
Streptomycin	10 μg	≥ 15	12–14	≤ 11
Cefotaxime	30 μg	≥26	23–25	≤ 22
Tetracycline	30 μg	≥ 15	12–14	≤ 11
Nalidixic acid	30 μg	≥ 19	14-18	≤ 13
Ciprofloxacin	5 μg	≥ 21	16–20	≤ 15
Trimethoprim-sulfamethoxazole	1.25/ 23.75 μg	≥ 16	11–15	≤ 10
Sulfonamides	250 or 300 μg	≥ 17	13–16	≤ 12
Trimethoprim	5 μg	≥ 16	11–15	≤ 10
Chloramphenicol	30 μg	≥ 18	13–17	≤ 12

3.2.5 Quality control

Every batch of prepared media was checked for sterility for 24 hours. *E. coli* ATCC 25922 was used as a quality control strain for antimicrobial susceptibility testing and screening and confirmatory tests for ESBLs in *E. coli*.

3.2.6 Statistical analysis

The data of the questionnaires and the laboratory results were analyzed using R statistic software (Hornik, 2013). General information on the farms and ESBL-producing *E. coli* prevalence were described. Descriptive statistic, including frequency, mean, standard deviation, median, minimum and maximum were employed as general analytic procedure to describe the general information on the farms, the occurrence of ESBLs or antimicrobial administration. The occurrence of the diseases or health problems in cows and calves were subjective notions of the frequency of the problems (rare = the problems are not happening very often; regular = the problems that frequently happen). The criteria for the usage of antimicrobials in the treatment of diseases or health problems were recorded as never (not at any occasion), sometimes (on some occasions), and frequent (on many occasions) (Kreausukon, 2011). Chi-square analysis was used to analyze the bivariate association between the positive and negative of ESBL-producing *E. coli* farms. Risk factors including herd administration (raised other animal together with cattle, feeding waste milk to calves, feeding antimicrobial residual milk to calves, Record for antimicrobial usage), antimicrobial usage (rotating use antimicrobials, Contineous use of antimicrobial until cured, Using slow release antimicrobials, Frequently use cephalosporin, Treat four teats instead of diseased teats for mastitis et al) associated with detection of ESBL-producing *E. coli* were analyzed also. The odds ratios (OR) and 95% confidence interval (CI) were calculated using R studio software for different risk factors. A P-value of < 0.05 was considered as statistically significant.