"Research Note"

Running title: Transmission of *Salmonella* by housefly

Transmission of *Salmonella* between swine farms by the housefly (*Musca domestica*)

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Abstract

Domestic pig is an important source of human salmonellosis and houseflies are potential mechanical vectors of food-borne *Salmonella* pathogens. In 2005, we recovered 144 *Salmonella* isolates from flies and swine stool samples from 11 farms in Taoyuan County and Hsin Chu County (northwestern Taiwan). A total of 71.5% of the isolates were resistant to at least three antibiotics. There were a total of 14 serotypes, and eight of these serotypes were present in both flies and swine stool samples. Multidrug resistant *Salmonella* with identical pulsed-field gel electrophoresis (PFGE) can be found between different swine farms. Among four common serotypes, we identified 18 PFGE patterns, eight of which were present in flies and swine stools. The similarity in PFGE profiles between isolates from swine and flies in different farms indicated the potential of flies to serve as a vector for transmission.

Flies can be vectors of various bacterial, protozoan, and viral pathogens. The U.S. Food and Drug Administration classifies the housefly (*Musca domestica*) as an important contributing factor in the dissemination of various infectious food-borne diseases, such as cholera, shigellosis, and salmonellosis (*14*). An estimated 1.4 million salmonellosis cases occur annually in the United States (*11*). Other studies have also identified houseflies as vectors or transporters of *Salmonella* (*8*). Flies may transmit bacteria via their sponging mouthparts, body and leg hairs, sticky parts of their feet, faecal deposition, or regurgitation or vomitus (*7*, *13, 15*).

Salmonella enterica is one of the most common causes of human food-borne infections and all serotypes of *Salmonella* are considered potential health hazards to humans (*16*). Most patients with *Salmonella* infections experience self-limited gastroenteritis and usually recover without treatment. However some invasive *Salmonella* infections can be life-threatening with hospitalization and antibiotic treatment required. Infection by multidrug resistant (MDR) *Salmonella* is associated with an increased rate of hospitalization, and such infections are difficult to treat (*3*). Currently, fluoroquinolones or third-generation cephalosporins are considered as drugs of choice. However, there are increasing reports of *Salmonella* resistance to these antibiotics (*17, 19*).

Swine salmonellosis is considered a significant public health problem. MDR or fluoroquinolone-resistant *Salmonella* isolated from swines and transmission of *Salmonella*

between different swine farms has been reported (*4*, *18*). In the present study, we collected *Salmonella* isolates from flies and swine stools at eleven swine farms in northwestern Taiwan to assess the potential of flies to serve as a vector for *Salmonella* transmission in different swine farms.

Materials and Methods

Sample collection. Eleven swine farms in Taoyuan (7 farms) and Hsin Chu County (4 farms) were randomly chosen for sampling in 2005 (Fig. 1). At each farm, 40 healthy swine fecal samples were collected and houseflies were collected from four pieces of Fly Trap paper (Duong Industry, Taiwan) that were placed on the feed trough or pigpen for 30 min. Five flies were random taken from each Fly Trap paper, and each fly sample isolated *Salmonella* separated. 220 fly samples and 440 swine stool samples were selected to isolated *Salmonella.*

***Salmonella* isolation, identification and serotyping.** Each fly sample and swine fecal sample (5 g) was added to 9 times volume of buffered peptone water (BPW; Difco, Detroit) and incubated at 37ºC overnight. Then, 0.1 mL sample was plated on modified semisolid Rappaport Vassiliadis (MSRV; Difco) medium and a 1 mL sample was added to 9.0 mL of tetrathionate broth (Difco) incubated at 42ºC for 24 h. Then, a loopful of selective

enrichment broth and the presumptive positive swarm zones on the MSRV medium were streaked on Xylose lysine deoxycholate agar (Difco) and brilliant green agar plates (Difco) and incubated at 37ºC overnight. The presumptive *Salmonella* colonies were selected and tested on triple sugar iron (TSI), lysine decarboxylase, citrate, and urease agars (Creative Microbiologicals, Taichung, Taiwan**)**, and examined with a slide agglutination test with polyvalent anti-*Salmonella* antisera to determine somatic antigens. The flagellar antigens were identified by a tube broth agglutination test. A phase reversal (phase inversion) process was performed by the paper-bridged method (*5*). The Kauffmann-White classification scheme was used to identify serovars. Commercial *Salmonella* somatic and flagellar antisera were purchased from S&A Reagents Lab (Bangkok, Thailand) and Denka Seiken (Tokyo, Japan), respectively.

Antimicrobial susceptibility testing. Antibiotic susceptibility was tested using the agar diffusion method, according to Clinical and Laboratory Standards Institute standards (CLSI; *6*). *Escherichia coli* ATCC 25922 was used as a control. The antimicrobial agents used were ampicillin (10 μg), amoxycillin/clavulanic acid (20/10 μg), chloramphenicol (30 μg), florfenicol (30 μg), trimethoprim-sulfamethoxazole (1.25/23.75 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), streptomycin (10 μg), gentamicin (10 μg), ceftriaxone (30 μg), and tetracycline (30 μg). Susceptible and resistant isolates were defined according to criteria

of the CLSI.

Genotyping by pulsed-field gel electrophoresis. Genotypes of all *Salmonella* Anatum, *Salmonella* Typhimurium, *Salmonella* Choleraesuis var. kunzendorf and *Salmonella* Derby isolates were characterized by pulsed-field gel electrophoresis (PFGE), following digestion of total genomic DNA by the restriction endonuclease *Xba*I (New England Biolabs, Inc., Beverly). The PFGE procedure was performed according to the standard protocol developed by the Centers for Disease Control and Prevention (*2*). The digested DNA was separated by use of CHEF DR II (Bio-Rad) in 0.5 × Tris-borate-EDTA at 14°C for 20 h. PFGE images were digitally recorded in tag image file format (TIFF) using a Kodak EDAS 290 (Eastman Kodak Co, NY, USA). Analysis of TIFF images was performed with GelCompare II® 5.0 software (Applied Maths, Belgium). Similarity between fingerprints was determined by the Dice correlation coefficient, with 1% band position tolerance. Dendrograms were generated by an unweighted pair group method using arithmetic average (UPGMA).

Results

A total of 58 flies (26.4%) and 86 swine fecal samples (19.5%) tested positive for *Salmonella*. For these *Salmonella*-positive samples, 48 flies and 60 fecal samples were from farms in Taoyuan County and 10 flies and 26 fecal samples were from Hsin Chu County.

For individual farms, the *Salmonella* positive rate of flies samples varied from 5% to 100%.

Our analysis of the *Salmonella* isolates indicated that the flies had 15 *Salmonella* serotypes and that the three predominant serotypes were *Salmonella* Anatum, *Salmonella* Choleraesuis var. kunzendorf, and *Salmonella* Derby. We identified 13 serotypes of *Salmonella* in swine feces, and the three predominant serotypes were *Salmonella* Anatum, *Salmonella* Derby, and *Salmonella* Typhimurium (Table 1).

All of the 144 *Salmonella* isolates were susceptible to ceftriaxone. All *Salmonella* isolates from swine were susceptible to enrofloxacin and ciprofloxacin. The resistance rates to the tested antibiotics were listed in table 2. 71.5% of the isolates were MDR (resistant to at least three antibiotics).

We performed PFGE to subtype 68 *Salmonella* Anatum isolates, 14 *Salmonella* Typhimurium, 9 *Salmonella* Choleraesuis var. kunzendorf, and 19 *Salmonella* Derby isolates (Table 3). The results indicated the presence of six PFGE profiles among the Anatum isolates (n = 68), with three of these isolates (AN2, AN3, and AN4) in flies and swine (Fig. 2). There were four PFGE profiles in the Choleraesuis var. kunzendorf isolates (n = 9), five PFGE profiles in the Derby isolates (n = 19), and three PFGE profiles in the Typhimurium isolates (n = 14). The CH3, DE3, DE4, TY1, and TY2 profiles were present in flies and stool samples. The index of discrimination is 0.854 (*9*).

Discussion

We also identified a high rate of antibiotic resistance in the *Salmonella* isolates collected in this study, especially from flies in Hsin-chu county. Only 9.02% of *Salmonella* isolates were sensitive to the 12 antimicrobial agents that we tested. The rate to all 12 antimicrobial agent resistance of *Salmonella* isolates suggests that these antimicrobial agents should be used in animal farms after careful consideration.

PFGE analysis of *Salmonella* isolates found that 8 PFGE profiles were presented in *Salmonella* both from flies and swine stools (AN2, AN3, AN4, CH3, DE3, DE4, TY1, and TY2). The AN2 profile was found in 5 swine farms. This result suggested that flies serve as vectors for *Salmonella* transmission. In fact, it is known that flies from environments contaminated with pathogens readily become contaminated themselves (*8*). Previous studies indicated that 19% of flies captured on broiler farms *(1)* and 22% flies captured in facilities that housed egg-laying hens were positive for *Salmonella (21)*. The Diptera is a large and complex order of arthropods, with worldwide distribution. Previous studies determined that the maximum flight distance of the house fly was 7 km (*12*) to over 20 km (*10*). Because of their affinity for decaying matter, garbage, and feces, flies have long been considered as vectors for bacteria in agricultural and urban environments and as a significant public health hazard. In particular, flies may function as temporary vectors of *Salmonella*, with the pathogen surviving for a longer period of time within the fly's body, with no adverse effect

upon the vector (*20*).

In conclusion, we used PFGE analysis to evaluate that house flies may function as vectors for *Salmonella* transmission between swine farms. In particular, it appears that houseflies can acquire, harbor, and transport over significant distances. These results suggest that implementation of an insect control program should be considered as part of a program to reduce some of the risk of *Salmonella* dissemination.

REFERENCES

1. Bailey, J.S., N. J. Stern, P. Fedorka-Cray, S. E. Craven, N. A. Cox, D. E. Cosby, S. Ladely, and M. T. Musgrove. 2001. Sources and movement of *Salmonella* through integrated poultry operations: a multistate epidemiological investigation. *J. Food Prot.* 64:1690-1697.

2. Barrett T. J., P. Gerner-Smidt, and B. Swaminathan. 2006. Interpretation of pulsed-field gel electrophoresis patterns in foodborne disease investigations and surveillance. *Foodborne Pathog. Dis.* 3: 20-31.

3. Boyd D., A. Cloeckaert, E. Chaslus-Dancla, and M. R. Mulvey. 2002. Characterization of variant *Salmonella* genomic island 1 multidrug resistance regions from serovars Typhimurium DT104 and Agona. *Antimicrob. Agents Chemother.* 46: 1714-1722.

4. Chen T. H., Y. C. Wang, Y. T. Chen, C. H. Yang, and K. S. Yeh. 2006. Serotype occurrence and antimicrobial susceptibility of *Salmonella* isolates recovered from pork carcasses in Taiwan (2000 through 2003). *J. Food Prot.* 69: 674-678.

5. Chiou C. S., J. F. Huang, L. H. Tsai, K. M. Hsu, C. S. Liao, and H. L. Chang. 2006. A simple and low-cost paper-bridged method for *Salmonella* phase reversal. *Diagn. Microbiol. Infect. Dis.* 54:315-317.

6. Clinical and Laboratory Standards Institute. 2006. Performance standards for antimicrobial disk susceptibility tests. Approved standard, 9th ed. Approved standard M2-A9. Clinical and Laboratory Standards Institute, Wayne, Pa.

7. Greenberg B., J. A. Kowalski, and M. J. Klowden**.** 1970. Factors affecting the transmission of *Salmonella* by flies: natural resistance to colonization and bacterial interference. *Infect. Immun.* 6**:**800-809.

8. Holt P. S., C. J. Geden, R. W. Moore, and R. K. Gast. 2007. Isolation of *Salmonella enterica* serovar Enteritidis from houseflies (*Musca domestica*) found in rooms containing *Salmonella* serovar Enteritidis-challenged hens. *Appl. Environ. Microbiol.* 73:6030-6035.

9. Hunter P. R. and M. A. Gaston. 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J. Clin. Microbiol.* 26:2465-2466.

10. Lysyk T. J., and R. C. Axtell. 1986. Movement and distribution of house flies (Diptera: Muscidae) between habitats in two livestock farms. *J. Econ. Entomol.* 79:993-998.

11. Mead P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro., P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* 5: 607-625.

12. Nazni W. A., H. Luke, W. M. Wan Rozita, A. G. Abdullah, I. Sa'diyah, A. H. Azahari, I. Zamree, S. B. Tan, H. L. Lee, and M. A. Sofian. 2005. Determination of the flight range and dispersal of the house fly, *Musca domestica* (L.) using mark release recapture technique. ***Trop. Biomed.*** 22:53-61.

13. Olsen A. R. 1998. Regulatory action criteria for filth and other extraneous materials. III. Review of flies and foodborne enteric disease. ***Regul. Toxicol. Pharmacol.*** 28:199-211.

14. Olsen A. R., J. S. Gecan, G. C. Ziobro, and J. R. Bryce. 2001. Regulatory action criteria for filth and other extraneous materials v. strategy for evaluating hazardous and nonhazardous filth. ***Regul. Toxicol. Pharmacol.*** 33:363-392.

15. Olsen A. R., and T. S. Hammack. 2000. Isolation of *Salmonella* spp. from the housefly, *Musca domestica* L., and the dump fly, *Hydrotaea aenescens* (Wiedemann) (Diptera: Muscidae), at caged-layer houses. ***J. Food Prot.*** 63**:**958-960.

16. Osterberg J., S. S. Lewerin, and P. Wallgren 2010. Direct and indirect transmission of four *Salmonella enterica* serotypes in pigs. ***Acta Vet. Scand.*** 52:30.

17. Rayamajhi N., S. G. Kang, D. Y. Lee, M. L. Kang, S. I. Lee, K. Y. Park, H. S. Lee, and H. S. Yoo. 2008. Characterization of TEM-, SHV- and AmpC-type beta-lactamases from cephalosporin-resistant *Enterobacteriaceae* isolated from swine. ***Int. J. Food Microbiol.*** 124:183-187.

18. Wang Y. C., Y. C. Chang, H. L. Chuang, C. C. Chiu, K. S. Yeh, C. C. Chang, S. L. Hsuan, and T. H. Chen. 2010. Antibiotic resistance, integrons and *Salmonella* genomic island 1 among *Salmonella* Schwarzengrund in broiler chicken and pig. ***Afr. J. Microbiol. Res.*** 4: 677-681.

19. Wang Y. C., K. S. Yeh, C. C. Chang, S. L. Hsuan, and T. H. Chen. 2006. Fluoroquinolone-resistant *Salmonella* sp. in carcasses. ***Emerg. Infect. Dis.*** 12:351-352.

20. West L. S. (ed.). 1951. The housefly. Its natural history, medical importance, and control. Comstock Publishing Co. Inc., New York.

Figure legends

FIGURE 1. Geographic location of the 11 swine farms in Taiwan where *Salmonella* isolates were collected in 2005. T1-T7 indicate farm locations in Taoyuan county and H1-H4 indicate farm locations in Hsin chu county .

FIGURE 2. Dendrograms of PFGE fingerprints of *Salmonella* Anatum (2A), *Salmonella* Choleraesuis var. kunzendorf (2B), *Salmonella* Derby (2C), and *Salmonella* Typhimurium (2D) isolates from swine and flies. Genetic similarity values between fingerprints were calculated based on Dice coefficient, with 1% band position tolerance. Dendrograms were generated by unweighted pair group method using arithmetic average (UPGMA).

Table 1. *Salmonella* serotypes isolated from flies and swine.

S. enterica serotype	Isolated from flies	Isolated from pigs	Overall
Anatum	25	42	67
Derby	6	13	19
Typhimurium	2	12	14
Schwarzengrund	4	7	11
Choleraesuis var. kunzendorf	7	2	9
Weltevreden	3	1	4
Enteritidis	2	1	3
Newport	1	1	2
Duesseldorf	0	2	2
Bardo	0	2	2
Bonn	0	1	1
Senftenberg	1	0	1
Gloucester	1	0	1
Agona	1	0	1
untypable	5	2	6
Total	58	86	144

Table 2. Differences in prevalence of *Salmonella* resistance to antimicrobials in *Salmonella* isolated from flies or swine.

Antibiotics	Isolated from flies			Isolated from pigs		
	Overall	Taoyuan	Hsin Chu	Overall	Taoyuan	Hsin Chu
Ampicillin	58.6%	54.2%	80.0%	54.7%	56.7%	50.0%
Amoxicillin-clavulanic acid	25.9%	25.0%	30.0%	3.5%	1.7%	7.7%
Ceftriaxone	0	0	0	0	0	0
Tetracycline	62.1%	54.2%	100%	79.1%	70.0%	100%
Streptomycin	58.6%	50.0%	100%	59.3%	51.7%	76.9%
Gentamicin	34.5%	20.8%	100%	37.2%	33.3%	46.2%
Sulphamethoxazole/Trimethoprim	48.4%	37.5%	100.%	60.5%	60.0%	61.6%
Chlormphenicol	62.1%	54.2%	100%	61.6%	65.0%	53.8%

Nalidixic acid	65.5%	58.3%	100%	65.0%	65.0%	50.0%
Enrofloxacin	31.0%	16.7%	100%	0	0	0
Ciprofloxacin	13.8%	12.5%	20.0%	0	0	0
Nitrofurantoin	48.3%	37.5%	100%	20.1%	10%	28.6%

Table 3 PFGE characterization of *Salmonella* isolates from flies and swine.

Serotype	PFGE pattern	Isolated from flies		Isolated from pigs	
		N	Farm	N	Farm
Anatum	AN 1	3	T3		
	AN 2	14	H4; T2, T3, T4, T6	24	H1, H2; T2, T3, T6
	AN 3	3	T3, T4	2	T1
	AN 4	6	T2. T3	4	T2
	AN 5			4	H2; T5,
	AN 6			6	H1; T8, T9
Choleraesuis var. kunzendorf	CH 1	3	T2		
	CH 2	1	T1		
	CH 3	2	T5	2	T5, T6
	CH 4	1	T7		
Derby	DE 1			2	H4
	DE 2			3	T7
	DE 3	4	T3, H3	5	T5
	DE 4	2	T4, T5	2	T5
	DE 5			1	T5

	TY 1	1	H3	5	H3; T1
Typhimurium	TY 2	1	T5	6	T4, T5
	TY 3			1	H4

Figure 1

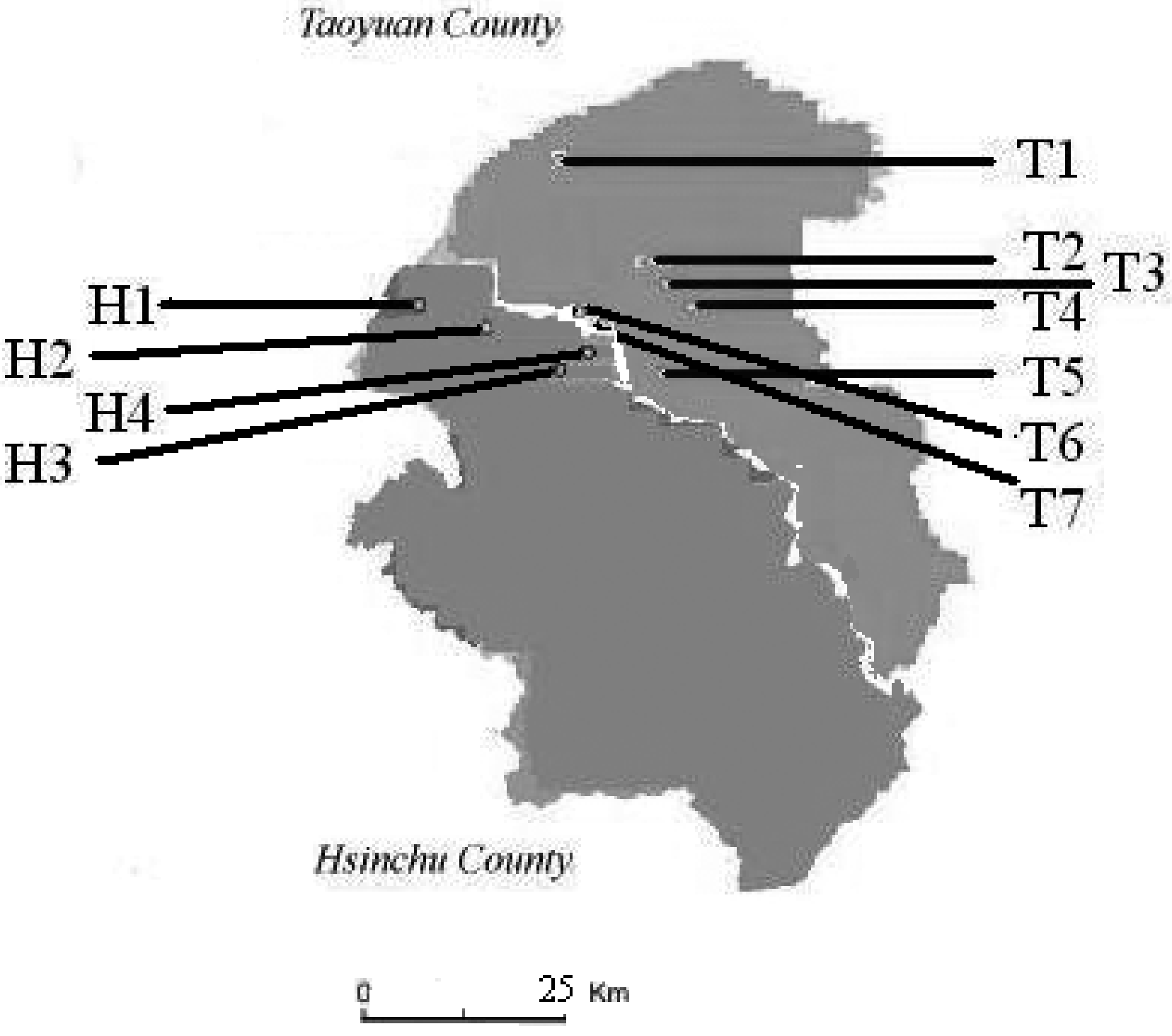
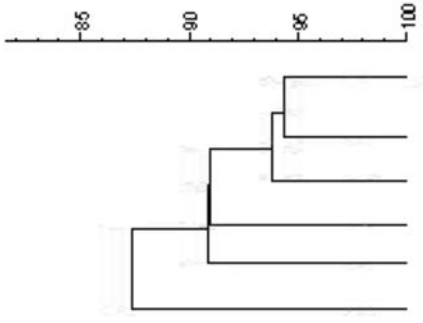
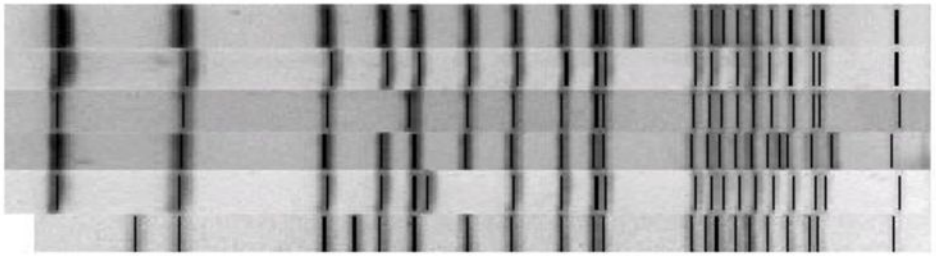

Taoyuan County
T1
T2
T3
T4
T5
T6
T7
H1
H2
H4
H3
Hsinchu County
0
25 Km

Figure 2

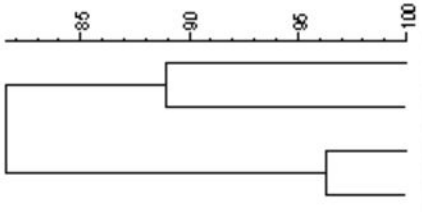
2 A

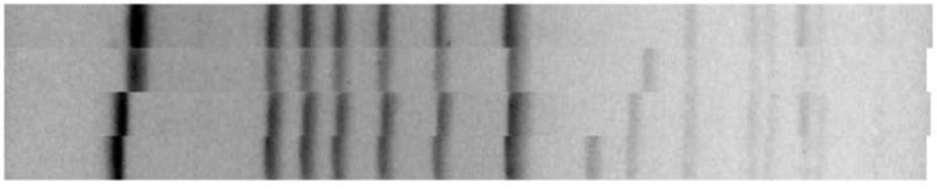




	From flies	From swine
AN 1	3	0
AN 2	14	24
AN 3	3	2
AN 4	6	4
AN 5	0	4
AN 6	0	6

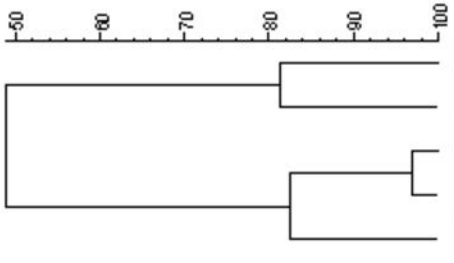
2B

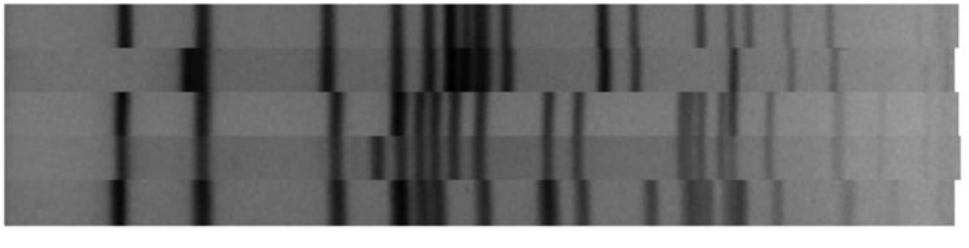




CH 1	3	0
CH 2	1	0
CH 3	2	2
CH 4	1	0

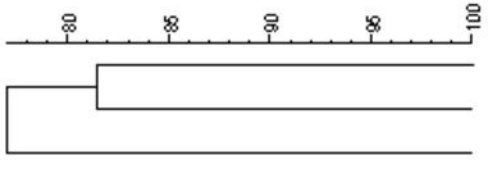
2C

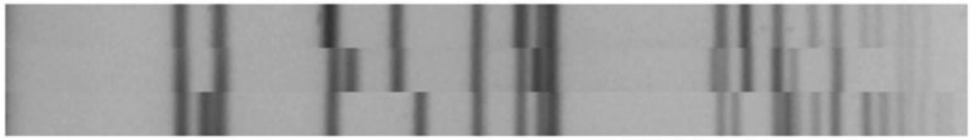




DE 1	0	2
DE 2	0	3
DE 3	4	5
DE 4	2	2
DE 5	0	1

2 D





TY 1	1	5
TY 2	1	6
TY 3	0	1