



## Increases of Antibiotic Resistance in Excessive Use of Antibiotics in Smallholder Dairy Farms in Northern Thailand

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**ABSTRACT:** Antibiotic resistance patterns of bacterial isolates from both quarter teat-tip swabs and their quarter milk samples were evaluated in smallholder dairy farms in northern Thailand with excessive use of antibiotics (HIGH) compared with normal use (NORM). Results from teat-tip swab samples showed that the percentage of *Bacillus spp.* resistance to overall antibiotics was significantly lower in the NORM group than that of the HIGH group, whereas, the resistance percentage of coagulase-negative staphylococci in the NORM group was higher than that of the HIGH one. The overall mastitis-causing bacteria isolated from milk samples were environmental streptococci (13.8%), coagulase-negative staphylococci (9.9%), *Staphylococcus aureus* (5.4%), and *Corynebacterium bovis* (4.5%). Both staphylococci and streptococci had significantly higher percentages of resistance to cloxacillin and oxacillin in the HIGH group when compared to the NORM one. An occurrence of vancomycin-resistant bacteria was also observed in the HIGH group. In conclusion, the smallholder dairy farms with excessive use of antibiotics had a higher probability of antibiotic-resistant pattern than the farms with normal use. (**Key Words:** Antibiotic Resistance, Environment, Mastitis, Cow, Bacteria, Dairy Farm)

### INTRODUCTION

In Southeast Asia, smallholder dairying has provided good income for crop farmers in mixed farming systems. With further improvement in productivity and reduction in production costs, Chantalakhana and Skunmun (2002) believe that smallholder dairying in this region can become a very sound and sustainable enterprise. Thailand has the fastest growing dairy industry in tropical Asia, with 610% more milk production in 2006 than in the period 1990/92; a growth rate of 38% per annum (Moran, 2009). In parallel with this rapid development, antibiotics are increasingly utilized in Thailand's dairy industries. Based on a report from the Thai Drug Control Division, the number of animal drugs produced in Thailand increased 3.9 times between 1996 and 2005 (Thai-Drug-Control-Division, 2009). The use of these antibiotics in animals and their accumulation in humans could lead to bacterial resistance, which would shorten the usable life of human medicines.

Antibiotics in dairy cattle are primarily used to treat or

prevent diseases and, hence, increase milk production and/or improve feed efficiency. No antibiotic is completely effective at eliminating all target bacteria within their complex microbial communities, which are frequently encountered within agricultural systems. Consequently, it is inevitable that antibiotic therapy will eventually reduce the number of antibiotic-susceptible strains and promote the development of antibiotic-resistant strains (McAllister et al., 2001). Studies in Thailand have found antibiotic resistance in some bacteria, especially *Salmonella spp.* isolated from food animals and humans (Boonmar et al., 1998; Hanson et al., 2002), including resistance to relatively new antibiotic agents such as azithromycin and ciprofloxacin (Isenbarger et al., 2002).

The use of antibiotics in animals in Thailand has been regulated by the Department of Livestock Development (DLD), Ministry of Agriculture and Cooperation, Thailand, since 2003. Nowadays, Thailand has successfully adopted developed country standards into a developing country framework since knowing that Thai poultry and seafood is inspected at EU customs. However, not all farms in the country have met these new requirements for antibiotic drug use. One tool for controlling antibiotic use is the

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certification of farm standards by the DLD. All antibiotic use in the standard dairy farms must be authorized by DLD trained and registered official-farm-advisor veterinarians, and recorded. Although the antibiotics used is an important component of a preventive and treatment program, the final decision for their use is taken by the farm owners themselves based on their experience and economic situation. The different frequency of antibiotic use might result in differences in the antibiotic resistance of bacteria in either the udder or environment. Therefore, the objective of this study was to identify the antibiotic resistance of pathogens isolated from either quarter milk or the environment.

## MATERIALS AND METHODS

### Farm selection and study design

This study was performed during 15th to 30th March 2008. Eighteen smallholder dairy farms (a farm with >20 milking cows) which were members of Mae Wang Dairy cooperative, Chiang Mai Province, Thailand, were included in the study. To control the difference of location, all farms were situated within a 5-km radius. All the farms had been certified as standard DLD farms for at least 2 years, and were advised by an official-farm-advisor veterinarian from their cooperative. All antibiotic use had been authorized by the veterinarian and recorded as a regulation of certified standard farms. All the farms had been in the herd health management program, including reproductive management, mastitis control program and data collection, for at least 6 months. The mastitis control program of their cooperative included monthly somatic cell count testing at farm and cow levels, decision making on the treatment of clinical and subclinical mastitis cows, regular milking machine instrument checking, price penalty on high bulk somatic cell count, and a fine of 20 times the cost of milk revenue when the milk sold was contaminated with antibiotics. Most cows were crossbred Holstein Friesian that were in free stall barns and milked twice daily. Bucket-type milking machines were used in all farms, with an average of 6 cows/milking unit.

The farms were separated into 2 groups based on their antibiotic use records by the authorized veterinarian. In general, Thai dairy farmers have an only routine use of antibiotic for antibiotic dry cow therapy, and most cows will experience once with antibiotic treatment during lactation. Therefore, most cows were received approximately 2 times in lactation with taking about 1 yr. From annual antibiotic use records, an annual average of antibiotic use in a farm was calculated from individual use, and was used to determine the farm group variable. The farm groups comprised i) normal use (NORM), being no more than 2 times/cow/yr of antibiotic use, and ii) excessive use (HIGH),

being more than 2 times/cow/yr. Milk samples were collected from chronic subclinical mastitis cows identified by <200,000 cells/cc of the last two somatic cell count (SCC) measurements or clinical mastitis cows at sample collection date. Milk samples, which were separated into quarters of subclinical mastitis cows, were aseptically collected. Systematic random sampling of cows selected with an odd-number was used to collect swab samples at the rear-right teat tip areas. Sterile cotton buds soaked in 0.9% normal saline were used to swab the 1×1 cm<sup>2</sup> area of the teat tips, and collect transport media. Samples were immediately transported to the milk quality laboratory of Faculty of Veterinary Medicine, Chiang Mai University.

### Bacterial identification

All milk samples were cultured on trypticase soy agar containing 5% bovine calf blood and incubated at 37°C for 48 h. The colonies presented were examined microbiologically according to standard procedures of the National Mastitis Council (Harmon et al., 1990). Criteria were made for the diagnosis of pathogens based on bacterial morphology, gram staining and biochemical tests. Distinguishing streptococci and staphylococci was carried out by the catalase test. Bacteria exhibiting a catalase positive reaction were examined further by the coagulase test to differentiate the potential of *Staph. aureus* from the usual coagulase-negative staphylococci (CNS), while catalase negative bacteria were tested with the CAMP reaction, esculin hydrolysis and sugar fermentation tests to distinguish *Streptococcus* spp. Grown gram-negative bacteria were also determined by using biochemical characteristics including, triple sugar iron agar, and motility indole lysine, citrate utilization and urea hydrolysis tests.

### Antibiotic susceptibility testing

Antibiotic susceptibility of the isolates was determined by the agar disc diffusion method according to the standard National Mastitis Council guideline (NMC, 1999). The antibiotics used included tetracycline (30 µg), oxacillin (1 µg), cloxacillin (5 µg), cephalexin (30 µg), vancomycin (30 µg), erythromycin (15 µg), gentamycin (10 µg), and neomycin (30 µg). Isolates categorized as susceptible or resistant (the latter including both “intermediate” and “resistant” isolates) were in accordance with the methods and criteria described by the National Committee for Clinical Laboratory Standards (NCCLS, 2002).

### Statistical analysis

Data on the distribution of bacterial isolates and antibiotic resistance were described by frequency and percentages. Comparisons of bacterial isolates and antibiotic susceptibilities were performed among the farm groups by Fisher’s Exact Chi-square tests. Significant levels

**Table 1.** Characteristics of farms with different antibiotics use: i) normal use (NORM), being no more than 2 times/cow/yr of antibiotic use, and ii) excessive use (HIGH), being more than 2 times/cow/yr

	NORM	HIGH	Statistic value	p-value
Farm characteristics				
Number of farms	8	10		
Total cows in farms	110	128		
Average cows per farm	13.75	12.80		
Subclinical mastitis prevalence in the last 3 months prior to study	0.43	0.35	4.53	0.04
Cow characteristics (mean±SEM)				
Average parity	2.66±0.23	3.71±0.34	-2.55	0.01
Average days in milk (days)	239±21.1	173±15.4	2.31	0.02
Geometric mean of somatic cells	845	580	1.65	0.10
Geometric mean of somatic cells in the last 3 months prior to study	387	361	0.21	0.83
Sample characteristics				
Number of subclinical mastitis cows	38	38	0.44	0.51
Number of sampling cows	36	37		
Number of quarter samples	140	147		
Positive intramammary-infected cows	23	24	0.00	1.00
Positive intramammary-infected quarters	50	56	0.09	0.77
Number of swab samples (cows)	12	15		

were defined at  $p < 0.05$ .

## RESULTS

The total number of milking cows from 18 smallholder dairy farms was 238, with an average of 13.22 cows/farms. Farms, cows and sample characteristics were separated into farm groups as described in Table 1. The general characteristics of farms and samples used in both groups were similar; except for the prevalence of subclinical mastitis in the NORM group during the last 3 months prior to study, which was significantly higher than that in the HIGH group. Cow performance in HIGH farms was different to that in NORM ones, including parity, days in

milk, and somatic cell counts (Table 1). From 38 subclinical cows in both groups, 2 in the NORM group and 1 in the HIGH one were excluded from experiments because of farm management problems. Four quarters in the NORM group and a quarter in the HIGH one did not function. Cows in the HIGH group had higher average parity and lower average days in milk than those in the NORM group ( $p < 0.05$ ).

Percentages of antibiotic resistance patterns of *Bacillus* spp. and CNS of the swab samples from farms with high and low antibiotic use are shown in Table 2. The percentages of all antibiotic resistance in *Bacillus* spp. were significantly lower in the NORM group (18.2%) than in the HIGH one (72.7%). In contrast, the percentage of CNS

**Table 2.** Percentages of antibiotic resistant patterns of *Bacillus* spp. and coagulase-negative staphylococci (CNS) from swab samples of farms with high (HIGH) and normal antibiotic use (NORM)

Antibiotics	<i>Bacillus</i> spp.				p	CNS				p
	NORM (n = 11)		HIGH (n = 11)			NORM (n = 8)		HIGH (n = 26)		
	S <sup>1</sup>	R <sup>1</sup>	S	R		S	R	S	R	
Oxytetracyclin	100.0	0.0	63.6	36.4	0.09	75.0	25.0	96.2	3.9	0.13
Cloxacillin	100.0	0.0	63.6	36.4	0.09	75.0	25.0	84.6	15.4	0.61
Oxacillin	100.0	0.0	72.7	27.3	0.21	75.0	25.0	84.6	15.4	0.61
Cephalexine	90.9	9.1	90.9	9.1	1.00	87.5	12.5	96.2	3.9	0.42
Vancomycin	100.0	0.0	90.9	9.1	1.00	100.0	0.0	96.2	3.9	1.00
Gentamycin	90.9	9.1	81.8	18.2	1.00	87.5	12.5	100.0	0.0	0.23
Erythromycin	100.0	0.0	81.8	18.2	0.48	87.5	12.5	96.2	3.9	0.42
Neomycin	90.9	9.1	90.9	9.1	1.00	87.5	12.5	88.5	11.5	1.00
All antibiotics <sup>a</sup>	81.8	18.2	27.3	72.7	0.03	37.5	62.5	76.9	23.1	0.08
Resistances $\geq 2$ antibiotics	90.9	9.1	54.6	45.5	0.15	75.0	25.0	80.8	19.2	1.00

<sup>1</sup> S = Susceptible, R = Resistance. <sup>a</sup> Resistant to at least one drug test.

**Table 3.** Bacterial isolates from quarter milk samples of subclinical mastitis cows separated into farm groups comprising i) normal use (NORM), being no more than 2 times/cow/yr of antibiotic use, and ii) excessive use (HIGH), being more than 2 times/cow/yr

	All		NORM		HIGH		p-value <sup>a</sup>
	N	Percent	N	Percent	N	Percent	
Negative sample	204	65.2	96	62.3	108	67.9	0.34
CNS	31	9.9	21	13.6	10	6.3	0.03
<i>Staph. Aureus</i>	17	5.4	13	8.4	4	2.5	0.02
<i>Strep. Uberis</i>	30	9.6	12	7.8	18	11.3	0.33
<i>Strep. Dysgalactiae</i>	10	3.2	7	4.6	3	1.9	0.21
<i>C. bovis</i>	14	4.5	0	0.0	14	8.8	<0.01
Other streptococci	3	1.0	2	1.3	1	0.6	0.62
Gram negative bacteria	4	1.2	3	2.0	1	0.6	0.74
Total	313	100.0	159	100.0	154	100.0	

<sup>a</sup> Comparisons of specified bacteria between HIGH and NORM.

resistance to all antibiotics in the NORM group (62.5%) was higher than that of the HIGH one (23.1%). Interestingly, two isolates were resistant to vancomycin in the HIGH group.

Bacterial isolates from quarter milk samples of subclinical mastitis cows were separated into farm groups as shown in Table 3. Overall, most isolates were environmental streptococci (13.8%), including *Strep. uberis* (9.6%), *Strep. dysgalactiae* (3.2%), and other streptococci (1.0%). Other isolates showing high levels of intramammary infection were CNS (9.9%), *Staph. aureus* (5.4%), and *C. bovis* (4.5%). Prevalence of CNS (13.6%) and *Strep. uberis* (11.3%) were at the highest level in the NORM and HIGH, respectively. In comparison between the two groups, the HIGH group had higher isolates of *C. bovis* ( $p < 0.05$ ), but significantly lower isolates of CNS and *Staph. aureus* than the NORM group.

Percentages of antibiotic resistant patterns of mastitis-causing pathogens from farms with high and normal antibiotic use are shown in Table 4. Values of overall resistance indicate that *Strep. uberis* and *Strep. dysgalactiae*

have high levels of resistance, especially to oxytetracycline, gentamycin and neomycin. In contrast, staphylococci were highly susceptible to oxytetracycline, gentamycin, or neomycin. The most susceptible antibiotic for all 4 mastitis pathogens were cephalaxine and vancomycin. These results suggest that there were differences in the percentage of isolates that showed resistance to cloxacillin between NORM and HIGH use farms. Resistance percentages of CNS, *Staph. aureus* and *Strep. uberis* against cloxacillin were higher in the HIGH group than in the NORM one. However, no significant difference was found in any statistical tests among the 4 mastitis pathogens in the NORM or HIGH group.

Percentages of antibiotic resistant patterns of staphylococci and streptococci, both of which cause mastitis, from farms with high and normal antibiotic use are shown in Table 5. Staphylococci were highly resistant to cloxacillin (28.6%) and oxacillin (21.0%) in the HIGH group, and streptococci were highly resistant to oxytetracycline (36.8% and 22.7%), gentamycin (73.7% and 77.3%) and neomycin (94.7% and 90.9%) in the

**Table 4.** Percentages of antibiotic resistant patterns of pathogens causing mastitis in farms with excessive and normal antibiotic use

Antibiotics	CNS		<i>Staph. aureus</i>		<i>Strep. uberis</i>		<i>Strep. dysgalactiae</i>	
	NORM (n = 21)	HIGH (n = 11)	NORM (n = 13)	HIGH (n = 4)	NORM (n = 12)	HIGH (n = 18)	NORM (n = 7)	HIGH (n = 3)
Oxytetracyclin	4.8	10.0	30.8	0.0	25.0	16.7	50.0	66.7
Cloxacillin	4.8*	10.0*	15.4*	25.0*	8.3*	22.2*	0.0	0.0
Oxacillin	9.5	20.0	0.0	25.0	0.0	16.7	0.0	0.0
Cephalaxine	0.0	0.0	0.0	0.0	0.0	5.6	0.0	0.0
Vancomycin	4.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Gentamycin	0.0	0.0	0.0	0.0	75.0	72.2	66.7	100.0
Erythromycin	9.5	0.0	0.0	0.0	0.0	5.6	0.0	0.0
Neomycin	0.0	10.0	0.0	0.0	91.7	77.8	100.0	100.0
All antibiotics <sup>a</sup>	23.8	45.5	38.5	25.0	91.7	88.9	100.0	100.0
Resistances $\geq 2$ antibiotics	4.8	18.2	0.0	0.0	25.0	22.2	28.6	66.7

<sup>a</sup> Resistant to at least one drug test. \* Comparisons of specified bacteria between HIGH and NORM.

**Table 5.** Percentages of antibiotic resistant patterns of staphylococci and streptococci, which both cause mastitis in farms with high and normal antibiotic use

Antibiotics	Staphylococci		Streptococci <sup>a</sup>		Both pathogens <sup>b</sup>	
	NORM (n = 34)	HIGH (n = 15)	NORM (n = 19)	HIGH (n = 22)	NORM (n = 55)	HIGH (n = 37)
Oxytetracyclin	14.7	7.1	36.8	22.7	21.8	16.2
Cloxacillin	14.7	28.6	5.3	22.7	10.9**	27.0**
Oxacillin	5.9*	21.0*	0.0	13.6	3.6**	18.9**
Cephalexine	2.9	7.1	0.0	4.5	1.8	5.4
Vancomycin	2.9	0.0	0.0	0.0	1.8	0.0
Gentamycin	0.0	0.0	73.7	77.3	27.3*	45.9*
Erythromycin	5.9	0.0	0.0	4.5	3.6	2.7
Neomycin	0.0	7.1	94.7	81.8	34.5*	51.3*
All antibiotics <sup>c</sup>	29.4	40.0	94.7	90.9	52.7*	69.4*
Resistance $\geq$ 2 antibiotics	2.9	13.3	26.3	31.8	10.9	22.2

<sup>a</sup> Streptococci including *Strep. uberis*, *Strep. dysgalactia*, and other streptococci. <sup>b</sup> Pathogens including both staphylococci and streptococci

<sup>c</sup> Resistant to at least one drug test. \* Differences between NORM and HIGH at  $p < 0.05$ . \*\* Differences between NORM and HIGH at  $p < 0.05$ .

NORM and HIGH group, respectively. Both staphylococci and streptococci were highly resistant to cloxacillin and oxacillin in the HIGH group. Most mastitis pathogens were susceptible to cephalexine, vancomycin and erythromycin. The percentage of antibiotic resistance for staphylococci against oxacillin in the NORM group was lower than that in the HIGH one. In comparison between pathogens of the HIGH and NORM group, antibiotic resistance against cloxacillin and oxacillin was associated with the frequency of antibiotic use in farms ( $p < 0.05$ ). Gentamycin, neomycin and all antibiotics in the HIGH group tended to have higher resistant percentages than in the NORM group.

## DISCUSSION

In this study, farms were separated into two groups: normal use (NORM), being no more than 2 times/cow/yr of antibiotic use, and excessive use (HIGH), being more than 2 times/cow/yr. Based on a report from Thai Drug Control Division, the number of animal drugs produced in Thailand increased 3.9 times between 1996 and 2005 (Thai-Drug-Control-Division, 2009). One of the food-safety issues in Thailand is misuse or over-use of antibiotics, which sometimes leaves drug residuals in meat and dairy products (Ranong and Chalermphao, 2003). Based on two consecutive SCC measurements, subclinical mastitis prevalence in the NORM group (0.43) was higher than that of the HIGH group (0.35) during the last 3 months prior study. The prevalence in this study was a little higher than that previously reported in Thailand (Chaikhun et al., 2005), and Ethiopia (Workineh et al., 2002; Almaw et al., 2008). Farms with high limitations on antibiotic use, especially organic farms, have a higher prevalence of subclinical mastitis than conventional farms (Roesch et al., 2007). A study conducted in Denmark provided strong evidence that the rate of

mastitis therapy was associated with a desire to reduce overall antibiotic treatment.

Public health concerns advise prudent use of antibiotics, as their use may promote bacterial antibiotic resistance and leave antibiotic residues in the food chain (Berry and Hillerton, 2002). To reduce the risk of getting diseases from antibiotic resistant bacteria, the use of antibiotics must be restricted. In this study, *Bacillus* spp. and CNS were used as bacterial normal flora in a dairy farm environment. *Bacillus* spp., including *B. cereus* and *B. anthracis*, were chosen as a representative for Gram-positive indigenous soil bacteria to test the effect of spreading to various environments in many studies (Jensen et al., 2001; Black et al., 2008). CNS was also used for investigating environment such as wastewater and drinking water (Faria et al., 2009). The percentages of *Bacillus* spp. resistance to all antibiotics in the NORM group were significantly lower than those of the HIGH group; whereas, the percentage of CNS resistance to all antibiotics the NORM group was higher than that of the HIGH one. Antibiotic resistant environmental bacteria, shown in this study, might relate to the high use of antibiotics in smallholder dairy farms in Thailand. In addition, vancomycin resistant bacteria were found in the HIGH group. The high use of antibiotic may participate in high frequency of improper antibiotic use resulting in increasing a frequency of antibiotic resistance (McAllister et al., 2001). In addition a conventional small dairy farm had lower antibiotic resistance after managing as an experimentally organic dairy farm for 6 month (Suriyasathaporn, 2010).

This study showed a similar result to a previous one in Chiang Mai Province, Thailand, in which most bacterial isolates were environmental streptococci and CNS (Boonyayatra et al., 2007; Suriyasathaporn, 2011). Regarding to antibiotic using, both CNS and *Strep. uberis*

were the most common isolates in the NORM and HIGH group, respectively. However, CNS is a member of the opportunistic pathogen group, which mostly causes subclinical mastitis and is, reported less on the problems of antibiotic resistance (Boonyayatra et al., 2007). Interestingly, *C. bovis* was isolated less in the NORM group than in the HIGH one ( $p < 0.05$ ). *C. bovis* is contagious mastitis-causing bacteria that are most commonly associated with subclinical infection. Intramammary infection of *C. bovis* not only causes moderately increasing SCC, but also mildly increasing California Mastitis Test or CMT score (Radostits et al., 2008). *C. bovis* might be related to fewer mastitis diagnoses, and is consequently found in the HIGH group.

Values of overall resistance indicate that *Strep. uberis* and *Strep. dysgalactiae* have high resistance levels, especially to oxytetracycline, gentamycin and neomycin (Table 4). In contrast, staphylococci are highly susceptible to oxytetracycline, gentamycin, or neomycin. All 4 mastitis pathogens were most susceptible to cephalaxine and vancomycin. In general, resistant isolates were distinguished by the difference of their minimal inhibitory concentration (MIC). Pol and Ruegg (2007) showed higher MIC levels of most antibiotics versus environmental streptococci than those found in CNS. In most antibiotic use in smallholder dairy farms, cloxacillin showed differences in percentages of antibiotic resistance between farms that used normal and high antibiotics. Percentages of CNS, *Staph. aureus* and *Strep. uberis* resistance against cloxacillin in the HIGH group were higher than those in the NORM one.

In northern Thailand, cloxacillin represents over 80% of the intramammary drugs available on the market. A wide variety of antibiotic drugs has been used, often in an indiscriminate and inappropriate manner, thus impairing solutions to problems or leading to aggravation. Moreover, an important problem that arises from this kind of conduct is the increasing occurrence of microbial resistance (Susamo and Ocampo, 1992). Furthermore, the wide use of sulfa-trimethoprim, tetracycline and gentamicin to treat gastro-intestinal and other diseases in cattle has probably aided in developing resistance to these antibiotic agents.

Cloxacillin is the antibiotic mostly used in Thailand's dairy farms and it showed differences in percentages of antibiotic resistance between the NORM and HIGH group. The percentages of CNS, *Staph. aureus* and *Strep. uberis* resistance to cloxacillin in the HIGH group were higher than those in the NORM group. However, no significant difference was found among the 4 mastitis pathogens in all statistical tests between the NORM and HIGH group. The lower usage of antibiotics in the NORM group might be related to lower resistance levels. A previous study showed a close relationship between levels of antibiotic resistance

and the exposition of antibiotic use (Lopez-Lozano et al., 2000).

## CONCLUSION

In conclusion, farms with excessive use of antibiotics had a lower prevalence of mastitis. Mastitis-causing bacteria occurring in farms with excessive use and normal use of antibiotics were environmental streptococci and coagulase negative staphylococci, respectively. The problem of antibiotic resistance was found more in farms with excessive antibiotic usage than those with normal usage.

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