Estimation of Interdependence Among Quarters of the Bovine Udder with Subclinical Mastitis and Implications for Analysis

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ABSTRACT

Interdependency among udder quarters with subclinical mastitis was evaluated on 150 farms using a total of 35,828 udder quarters. The occurrence of high somatic cell count (SCC) (>250,000 cells/ml) in 0, 3, and 4 quarters occurred at a higher rate than would be expected based on independence of the quarters. For all bacterial species, intramammary infection in 0, 2, 3, or 4 quarters of the same cow occurred at a higher rate than would be expected based on independence of the quarters. Intramammary infection and high SCC were found less often in front quarters than in rear quarters. High SCC and intramammary infection occurred more often in right front quarters than in left front quarters. High SCC in diagonal quarters occurred at a lower rate than expected. *Corynebacterium bovis*, *Streptococcus agalactiae*, and *Staphylococcus aureus* had the highest intraclass correlation within herd. *Streptococcus uberis* had a very low intraclass correlation within herd. The intraclass correlation within cow for the natural logarithm of SCC was 0.47. *Corynebacterium bovis* and *Strep. agalactiae* had the highest intraclass correlation within cow, and *Streptococcus dysgalactiae* had the lowest. Analytical methods were proposed to manage the problem of interdependence and its effect on the design or evaluation of field studies on subclinical mastitis.
(**Key words**: somatic cell count, mastitis, interdependence, quarter)

Abbreviation key: **BMSCC** = bulk milk SCC, **GEE** = general estimation equations, **ICC** = intraclass correlation, **VIF** = variance inflation factor.

INTRODUCTION

A considerable amount of research has been devoted to the study of subclinical mastitis, and, based on this research, guidelines have been developed to evaluate experimental data. Current guidelines to evaluate clinical and subclinical infection status have been based on the assumption that the quarters within a cow are independent and that an equal probability exists that either the left or the right quarters could be infected (8). However, cross-infections of pathogenic bacteria within cows have been described. A study by Adkinson et al. (1) proved that the quarters within a cow are more alike with regard to susceptibility to clinical mastitis than would be expected based on independence of the quarters. Those researchers (1) observed that diagonal pairs of quarters with clinical mastitis occurred less often than expected. The incidence of clinical mastitis was found to be higher in rear quarters than in front quarters (1, 2). Most studies (1, 2) did not show a difference in incidence of clinical mastitis between right and left quarters. However, Walsh (23) reported different prevalence of high SCC and incidence of clinical mastitis between right and left quarters for a herd with a one-sided milking parlor.

Grootenhuis (6) and Flock and Zeidler (5), in studies that evaluated interdependence of quarters with subclinical mastitis, observed that cows with no infected quarters or 4 infected quarters were found more frequently than expected based on independence of the quarters. Front quarters had fewer IMI than did rear quarters, and diagonally infected pairs occurred less often than expected. The interdependence of quarters for IMI caused by *Staphylococcus aureus* has also been described (11).

Interdependence of quarters is based either on similar risk factors of quarters within a cow or within a herd or on contagiousness of microorganisms. When

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several quarters are infected in the proximity of an uninfected quarter, risk of IMI in the uninfected quarter is high.

When interdependence is ignored and when quarters are treated as independent observations, statistical significance tests may be unreliable. Specifically, tests may lead to an underestimation of Type 1 error because variance is underestimated. Additionally, knowledge of the clustering of an IMI, either within a cow or within a herd, may be of considerable interest and may lead to further understanding of the dynamics of the disease. Interdependence based on contagiousness may also lead to an underestimation of treatment effects (11).

Differences between the prevalence of subclinical mastitis at the cow and quarter levels can be corrected using intraclass correlation (**ICC**), which describes the strength of clustering (4). However, stable estimates of ICC for subclinical mastitis and IMI caused by specific mastitis pathogens are not available.

The purpose of this study was to evaluate the interdependence of quarters for subclinical mastitis that is caused by different pathogens and to propose methods to deal with the problem of interdependence when field studies on subclinical mastitis are designed or evaluated.

MATERIALS AND METHODS

Farms

Based on mean bulk milk SCC (**BMSCC**), three groups of 50 dairy farms were selected. In order for a farm to be selected, during 10 of 13 times during the previous year and during the final three times that BMSCC was evaluated, the SCC had to be in one of the following three categories: ≤150,000, 151,000 to 250,000, or 251,000 to 400,000 cells/ml. These three categories were selected to include relevant ranges of BMSCC under current farming conditions in The Netherlands. Selected farms housed the lactating cows in a free-stall barn, participated in a milk recording system, had a production quota of at least 300,000 kg, and had cows of the Holstein-Friesian or Dutch Friesian breeds. Milking parlors were double herringbone or a two-sided open tandem shape.

Sampling

Foremilk samples were collected from all quarters of all lactating cows in the 150 herds in a 1.5-yr period starting in December 1992. After the first streams of milk were discarded, teat ends were disinfected, and quarter foremilk samples were collected. When a cow was sampled from her right side, the sampling order of the quarters was right front, right rear, left front, and left rear. When a cow was sampled from her left side, the sampling order was left front, left rear, right front, and right rear.

Laboratory Procedures

The samples were stored at approximately 5°C immediately after milking. The samples were split into two portions, and SCC were determined by a Fossomatic cell counter (Foss Electric, Hillerød, Denmark) within 16 h. No preservative was used. The remainder of the milk sample was stored at –20°C before bacteriological culturing. After the freezing period, the milk samples were thawed at room temperature (20°C) for bacteriological analysis.

Bacteriological culturing of milk samples was performed according to standards of the National Mastitis Council (7); 0.01 ml was cultured. In each culture, the number of colony-forming units of each bacterial species was counted. A quarter was considered to have an IMI when ≥500 cfu/ml of a bacterial species were found. A quarter was defined as a high SCC quarter if the SCC was ≥250,000 cells/ml (22). When only 3 of the 4 quarters were sampled, the cow was excluded from the study as not being at risk for having 4 infected quarters or for having a high SCC. Farms on which no *Streptococcus agalactiae* was isolated were excluded from the analysis concerning this species.

Statistical Analysis

The pooled data were used to determine the distribution of IMI and high SCC among quarters in the udder. Prevalence of IMI and high SCC, categorized according to the number of quarters within a cow, were compared with expectations based on the assumption that quarters were independent. A binomial probability distribution was used to determine the expected number of infected quarters per cow using the following formula (4):

$$P(x) = \binom{4}{x} P_r^{x} (1 - P_r)^{4 - x} \quad [1]$$

where $P(x)$ = probability that x of the four quarters would have an IMI or a high SCC, and P_r = overall proportion of quarters with an IMI or a high SCC.

Prior to statistical analyses, data were checked for unlikely values; no data were excluded for this reason. Missing values routinely caused a record to be

excluded if the analysis included that variable. Statistical significance was defined at $P = 0.05$. The distribution of high SCC and IMI across quarters was determined using the frequency procedure of SAS (16). Differences between observed and expected distributions of IMI and high SCC across quarters were tested using chi-square analysis.

Variance components of herd and of cow within a herd were estimated. To approximate the normal distribution, a natural logarithmic transformation of the SCC (1000 cells/ml) was used (21). Data were analyzed using the MIVQUE method in the VARCOMP procedure of SAS (16). The model considered herd and cow nested within herd. Additionally, the ICC were estimated with both herd and cow as classification factors. The ICC within cow (ICC_c) and within herd (ICC_h) were defined using Equations [2] and [3], respectively (14, 20):

$$ICC_c = \frac{\sigma_c^2}{(\sigma_c^2 + \sigma_e^2)} \text{ and} \quad [2]$$

$$ICC_h = \frac{\sigma_h^2}{(\sigma_h^2 + \sigma_c^2 + \sigma_e^2)} \quad [3]$$

where σ_c^2 = cow variance components, σ_h^2 = herd variance components, and σ_e^2 = residual variance components.

RESULTS

Description of the Data

The mean herd size of the 150 farms evaluated in this study was 61 lactating cows (SD = 18.1) and varied from 29 to 117 cows. Two hundred forty-eight cows had 3 or fewer quarters in production and were excluded from the analyses. For 25 cows, the SCC of at least 1 quarter was missing, and these cows could not be included in any analysis concerning SCC. Of the remaining 35,728 quarters, a total of 8254 (23.1%) were considered to have a high SCC (Table 1); at least 1 quarter of 46.9% of the cows had a high SCC.

Of the major pathogens, *Staph. aureus* was most frequently isolated (Table 2). *Streptococcus agalactiae* was diagnosed on 16 farms. Because *Escherichia coli* was found in only 54 quarters (35 rear and 19 front) and because *Actinomyces pyogenes* was detected in only 18 quarters (12 rear and 6 front), the prevalences of IMI associated with these two pathogens were considered too low to be included in the analysis. *Corynebacterium bovis* and coagulase-negative staphylococci were the most frequently isolated minor pathogens (Table 3).

TABLE 1. Distribution of quarters per cow (n = 8932) with SCC >250,000 cells/ml.

Quarter[1]	Observed		Expected	
	(no.)	(%)	(no.)	(%)
0	4742	53.1	3123	35.0
1	1926	21.6	3753	42.0
2	1068	12.0	1691	18.9
3	592	6.6	339	4.0
4	604	6.8	25	0.3
RF	416	4.7	482	5.4
LF	300	3.4	482	5.4
RR	618	6.9	482	5.4
LR	592	6.6	482	5.4
RF and LF	173	1.9	178	2.0
RF and RR	188	2.1	178	2.0
RF and LR	132	1.5	178	2.0
LF and RR	117	1.3	178	2.0
LF and LR	163	1.8	178	2.0
RR and LR	295	3.3	178	2.0

[1]RF = Right front, LF = left front, RR = right rear, and LR = left rear.

Within-Cow Effect

Quarters within a cow were more alike with respect to high SCC than would be expected based on independence of the quarters. High SCC in 4, 3, or 0 quarters occurred at a higher rate than expected ($P < 0.00001$; Table 1).

Quarters within a cow were more alike with regard to IMI than would be expected based on independence of the quarters ($P < 0.00001$; Tables 2 and 3). For all bacterial species, IMI in 3 or 4 quarters of the same cow occurred at a higher rate than expected. Except for coagulase-negative staphylococci and *C. bovis*, the same result was found concerning IMI in 2 quarters within a cow for all bacterial species ($P < 0.00001$). For all bacterial species, cows with only one infected quarter were fewer than expected ($P < 0.00001$). Cows with no infected quarters were found more frequently than expected ($P < 0.00001$).

Differences Among Quarters

Front quarters had a high SCC less often than did rear quarters ($P < 0.00001$; Table 1). High SCC in right front quarters occurred more often than high SCC in left front quarters ($P < 0.01$; Table 1). High SCC in two diagonal quarters occurred at a lower rate than expected ($P < 0.001$; Table 1).

TABLE 2. Distribution of the major pathogens in quarters per cow (n = 8957).

	Streptococcus dysgalactiae		*Streptococcus agalactiae*[1]		*Streptococcus uberis*		*Staphylococcus aureus*	
Quarter[2]	O[3]	E	O	E	O	E	O	E
0	8410	8320	901	862	8757	8714	7798	7523
1	463	620	48	110	168	240	878	1341
2	62	17	19	5	22	2	206	90
3	20	0	5	0	7	0	61	3
4	2	0	5	0	3	0	14	0
RF	97	116	10	12	36	42	219	220
LF	106	116	9	12	25	42	184	220
RR	136	116	16	12	49	42	245	220
LR	124	116	13	12	58	42	230	220
RF and LF	4	10	4	3	6	4	32	34
RF and RR	14	10	1	3	0	4	30	34
RF and LR	8	10	6	3	1	4	26	34
LF and RR	11	10	2	3	4	4	37	34
LF and LR	10	10	3	3	3	4	33	34
RR and LR	15	10	3	3	8	4	48	34

[1]Only farms with isolates of *Strep. agalactiae* were included.

[2]RF = Right front, LF = left front, RR = right rear, and LR = left rear.

[3]O = Observed; E = expected.

Left front and left rear quarters had IMI less often than did right front and right rear quarters, respectively ($P = 0.03$ and $P = 0.01$, respectively; Table 4); the same result was true when right front quarters were compared with right rear quarters ($P = 0.002$) and when left front quarters were compared with left rear quarters ($P = 0.05$; Table 4). For *Streptococcus dysgalactiae*, *Streptococcus* spp. group D, *Streptococcus uberis*, *Staph. aureus*, coagulase-negative staphylococci, and *Bacillus* spp., the frequency of isolation was higher ($P < 0.05$) in rear quarters than in front quarters (Table 4). For *Strep. dysgalactiae*, *Staph. aureus*, *Streptococcus* spp. group D, and *Bacillus* spp., the same relationship between left and right quarters was also found when 2 infected front quarters were compared with 2 infected rear quarters ($P < 0.05$; Tables 2 and 3). Diagonal pairs of quarters infected with coagulase-negative staphylococci and *C. bovis*

TABLE 3. Distribution of the minor pathogens in quarters per cow (n = 8957).

	Streptococcus spp. Lancefield group D		Other streptococci		Coagulase-negative staphylococci		*Corynebacterium bovis*		*Bacillus* spp.	
Quarter[1]	O[2]	E	O	E	O	E	O	E	O	E
0	8114	7935	7903	7661	5798	4891	3849	2203	8606	8488
1	669	976	797	1221	1901	3196	1960	3701	257	459
2	132	45	202	73	771	783	1437	2331	65	9
3	32	1	48	2	361	85	1082	653	25	0
4	10	0	7	0	126	3	629	69	4	0
RF	152	167	210	199	451	475	528	490	61	64
LF	123	167	181	199	380	475	453	490	46	64
RR	209	167	189	199	548	475	527	490	73	64
LR	185	167	217	199	522	475	452	490	77	64
RF and LF	11	22	40	34	157	129	274	240	11	11
RF and RR	19	22	37	34	133	129	269	240	6	11
RF and LR	17	22	23	34	103	129	210	240	8	11
LF and RR	26	22	42	34	101	129	202	240	13	11
LF and LR	25	22	27	34	112	129	199	240	9	11
RR and LR	34	22	33	34	165	129	283	240	18	11

[1]RF = Right front, LF = left front, RR = right rear, and LR = left rear.

[2]O = Observed; E = expected.

were found less frequently than expected based on independence of quarters ($P < 0.01$; Table 3).

ICC

Of the major pathogens, *Strep. agalactiae* and *Staph. aureus* had the highest ICC within herd (Table 5). Of the minor pathogens, *C. bovis* showed the highest ICC within herd. *Streptococcus uberis* had the lowest ICC within herd.

The ICC within cow for the natural logarithm of SCC was 0.47, indicating a strong correlation of SCC among quarters of the same cow (Table 5). Of the major pathogens, *Strep. agalactiae* had the highest ICC within cow, and *Strep. dysgalactiae* had the lowest ICC within cow. *Corynebacterium bovis* also had a high ICC within cow.

DISCUSSION

Our data showed a strong interdependence between IMI and high SCC of quarters both within herd and within cow. The ICC within herd ranged from 0.01 to 0.11; ICC within cow ranged from 0.08 to 0.28. The difference in ICC among pathogens was considerable. Differences in ICC among herds were especially apparent for known contagious pathogens such as *Strep. agalactiae* (3), *C. bovis*, coagulase-negative staphylococci, and *Staph. aureus* (11). Differences in prevalence among herds might have been caused by differences in hygiene and management practices (15). These practices, however, are less adequate to prevent IMI that are caused by environmental pathogens such as *Strep. uberis* (18).

The observed interdependence of quarters within a cow could be attributed to general cow effects such as milk yield and immune competency. Additionally, pathogen-specific effects, such as transmission patterns of infection, should be considered.

Transmission of IMI occurs not only among cows but also among quarters within a cow. Because known contagious pathogens such as *Strep. agalactiae* (3) and *Staph. aureus* (11) are mainly transmitted during milking (including preparation before milking), interdependence among quarters based on transmission is more likely for these pathogens than for pathogens such as *Strep. dysgalactiae*, *Strep. uberis*, and *E. coli*. Coagulase-negative staphylococci and *C. bovis* are contagious minor pathogens (12).

The difference in infection status that was found between left and right quarters has implications for the design of experimental studies. If not corrected, this difference could lead to biased estimates of effect, especially in studies using a split-udder design. This problem can be solved by a random selection of treatment and control quarters (19) or by using a random block design (10). In this study, sampling order of quarters within a cow was not the origin of the difference; equal numbers of cows were sampled from the right and left sides because of the construction of the milking parlors.

In the case of subclinical mastitis, the interdependence of quarters should be taken into consideration when experiments are designed and evaluated, which is particularly important for the evaluation of dry cow products, lactation treatments, and teat disinfection products. In such studies, which use measurements taken from individual quarters, ignoring interdependence among quarters could lead to a serious underestimation of variance and an associated increase of Type 1 error. In split-udder trials, the assumption of

TABLE 4. Distribution of infected quarters per pathogen in 35,828 quarters of 8957 cows.[1]

	RF		LF		RR		LR		Total	
	O[2]	E	O	E	O	E	O	E	no.	%
Streptococcus dysgalactiae	141	164	147	164	194	164	173	164	655	1.83
Streptococcus spp. Lancefield group D	229	267	219	267	321	267	300	267	1069	2.98
Streptococcus agalactiae[3]	31	30	27	30	30	30	33	30	121	3.09
Streptococcus uberis	50	61	45	61	70	61	80	61	245	0.68
Other streptococci	349	343	335	343	347	343	342	343	1373	3.83
Staphylococcus aureus	366	382	348	382	421	382	394	382	1529	4.27
Coagulase-negative staphylococci	1259	1258	1125	1258	1357	1258	1289	1258	5030	14.04
Corynebacterium bovis	2746	2649	2555	2649	2752	2649	2543	2649	10,596	29.57
Bacillus spp.	110	120	101	120	132	120	135	120	478	1.33
All[4]	4022	3987	3745	3987	4216	3987	3965	3987	15,948	44.64

[1]RF = Right front, LF = left front, RR = right rear, and LR = left rear.

[2]O = Observed; E = expected.

[3]Only farms with isolates of *Strep. agalactiae* were included.

[4]Number of quarters infected with at least one pathogen.

TABLE 5. Variance components and intraclass correlation (ICC) of infected quarters per cow.

	Herd	Cow within herd	Error	ICC	
				Within herd	Within cow
Natural logarithm of SCC	0.173973	1.339574	1.517270	0.06	0.47
Streptococcus dysgalactiae	0.000602	0.001561	0.015787	0.03	0.09
Streptococcus Lancefield group D	0.001583	0.002896	0.024477	0.05	0.11
Streptococcus agalactiae	0.000329	0.000852	0.002215	0.10	0.28
Streptococcus uberis	0.000073	0.001016	0.005703	0.01	0.15
Other streptococci	0.003053	0.002718	0.031101	0.08	0.08
Staphylococcus aureus	0.002229	0.004763	0.033876	0.05	0.12
Coagulase-negative staphylococci	0.008797	0.020141	0.091822	0.07	0.18
Corynebacterium bovis	0.023866	0.046445	0.138183	0.11	0.25
Bacillus spp.	0.001489	0.001394	0.010290	0.11	0.12

independence of quarters could lead to an underestimation of treatment effects because of the contagiousness of microorganisms.

Implications of Interdependence for Mastitis Trials

The choice of methods to account for interdependence depends on two main considerations: the objectives of the study and the assumptions that can be made about the nature of the correlation structure (14). If the study objectives include the investigation of factors at both the quarter and cow level, or even on the herd level, some type of statistical control is required. However, if the study objectives are to test treatment efficacy and no inferences are to be made on risk factors for IMI or clinical mastitis at the cow or herd level, a more effective control of cluster effects can be achieved by the design of the study. One method to avoid some of the problems of ICC within cow when the udder quarter is the unit of interest is to match case quarters with control quarters within the same cow. This procedure allows the investigator to focus on individual quarter risk factors and to eliminate cow effects (14). In clinical trials, this method can be applied using a split-udder design (19). Interdependence because of contagiousness of pathogens is not eliminated but can be corrected for in this design (11).

The two fundamental problems when interdependence is present are underestimation of variance (12, 14) and bias in effect estimation (11). Some methods can correct for interdependence among observations in the analysis; variance inflation factor (**VIF**) and general estimation equations (**GEE**) methods can be used when the major goal is to reduce underestimation of variance.

The use of VIF is a straightforward way to correct for variance for interdependence in the analysis of the data (14). The calculated variance must then be multiplied by this VIF: robust variance = VIF $\times$ simple variance. The VIF is estimated as VIF = $1 + [(n - 1) \times ICC]$ where n = mean cluster size. Because of the size of our study population, stable estimates of pathogen-specific ICC were obtained from our data. These estimates may be used in other trials if the nature of clustering (e.g., through milking machine) is similar to that used in our study. If it is assumed that all cows have 4 quarters in production, VIF = $1 + (3 \times ICC)$. The ICC for each pathogen is presented in Table 5. As an example, the results of a study of Hogan et al. (9) are discussed. The prevalence of IMI in 16 herds using four different teat disinfection practices was studied. The mean number of cows per farm (cluster size) was 57. Those researchers (9) found an 8.5% (SD = 3.4; variance $\cong$ 11.6) quarter prevalence of IMI caused by *Staph. aureus* in 4 herds that did not practice teat disinfection compared with a prevalence of 1.6% (SD = 1.3; variance $\cong$ 1.7) in 4 herds that used an iodophor teat dip as a postmilking teat disinfectant. The VIF within herd for *Staph. aureus* for that study (9) would be VIF = $1 + (56 \times 0.05) = 2.8$. The VIF within cow for *Staph. aureus* for that study (9) would be VIF = $1 + (3 \times 0.12) = 1.36$. The VIF, corrected for both within-cow and within-herd clustering, would be VIF = $2.8 \times 1.36 = 3.81$, which leads to a substantial increase in variance. Results of a significance test would be affected by this correction.

Statistical models that account for clustering and simultaneously estimate effects have recently become available. Simultaneous estimation is preferable because covariates that are included in the analysis may actually explain part of the correlation that is present in the raw data. For example, part of the within-cow clustering of IMI could be explained by age, stage of lactation, or other diseases. Hence, the actual ICC that is present in the data after correction for these covariates is substantially smaller than the ICC estimated from the raw data.

For binomially distributed data, the regression models that allow for simultaneous estimation are based either on a transformation of the outcome variable and subsequent application of techniques that assume normal distribution (17) or on models that allow for ICC and preserve the original distribution of the data. Zeger et al. (25) introduced GEE that are an example of the latter strategy. A number of error distributions, including the binomial, and a number of link functions, including the logistic function, are allowed. These GEE methods are then comparable with simple logistic regression but allow simultaneous inclusion of correlation structures among observations. Several correlation structures have been proposed, including autoregressive, m-dependent, compound symmetry, or an unspecified structure. The assumption of an equal correlation among all quarters is applicable for the correlation of quarters within a cow, which is likely to be a compound symmetry structure. This latter method was used by Leslie et al. (13) in the analysis of data regarding dry cow treatment. The ICC estimated from our data may be used as an initial estimate of correlation structure in GEE.

The limitation of GEE is that multilevel models cannot easily be fitted, which may occur when ICC is present at the cow level as well as at the herd level. For multiple levels of clustering, a linearized approach, such as Schall's algorithm, or a generalized linear mixed models procedure (24), such as the model applied in the macro GLIMMIX in SAS (16), may be utilized. These models allow the inclusion of multiple levels of correlation among observations. No applications of these latter methods using udder health data have been published to date.

In summary, VIF methods can be used to correct variance after the estimation of effects, and GEE or similar methods can be applied simultaneously with effect estimation. The previously mentioned methods mostly eliminate the underestimation of variance. Underestimation of efficacy because of contagiousness of microorganisms may still be present. This contagiousness may be modeled, and efficacy may be corrected.

The transfer of pathogens among cows and among quarters within a cow may occur. When the pattern of infection among cows can be modeled, the correlation of quarters within a cow is not a nuisance factor, but instead becomes the main interest of the modeling procedure. A model to describe the effect of the number of existing IMI on the number of new IMI has been described by Lam et al. (11). An adjusted model for contagious disease was applied to the dynamics of IMI caused by *Staph. aureus* within and among cows. The described modeling procedure allowed estimation of effects of covariates such as parity and days in lactation. Possible biases in the effect estimation were then corrected in the analysis.

CONCLUSIONS

When the prevalence of IMI or subclinical mastitis is studied at the animal or herd level and when measurement is made at the quarter level, correction for interdependence has to be considered. Statistical methods to correct for the underestimation of the variance are available.

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