Determination of Twin Zygosity: A Comparison of DNA with Various Questionnaire Indices

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This study examined cross-validation and test-retest reliability of questions and questionnaire indices commonly used for twin zygosity classification. Mothers of 58 monozygotic (MZ) and 52 dizygotic (DZ) same sex twin pairs were interviewed by telephone to answer questions regarding the similarity of their twins (mean age = 14.6 ± 2.8 years). A logistic regression equation correctly classified 91% of both MZ and DZ twin pairs in our sample using 7 of the 12 zygosity questions. The internal consistency for the total questionnaire (Cronbach's alpha) was 0.88. The median two month temporal stability estimate for the individual questions was r = .56 and r = .79 for the test total. For the cross-validation, zygosity classification indices taken from 9 previous studies were applied to our sample and compared to classification according to DNA microsatellite analyses (agreement range = 44 to 100%). The accuracy of the classification indices was significantly lower than the original studies for 62% of the comparisons. If zygosity determination with DNA markers or blood group typing for all subjects is not feasible, rather than using classification indices based on other studies, an optimal classification scheme can be achieved by using a zygosity questionnaire of which the reliability and validity of the questions is established in a random subsample of the same twin cohort.

Classical twin studies rely upon accurate zygosity classification. Incorrect classification may have a potentially large effect on heritability estimates, particularly for small twin cohorts. A classic study by Smith and Penrose (1955) provided a method for computing the probability of a correct classification for same sex DZ twin pairs based upon combinations of blood group systems. Wilson (1980) and Lykken (1978) each demonstrated that serological systems could provide highly accurate zygosity classification (97–98%) when a sufficient number of markers are used. DNA analysis using polymorphic DNA microsatellite markers has also been shown to provide highly accurate zygosity determination (98–99%) (Becker et al., 1997; Porrini et al., 1990). Dermatoglyphic studies generally have yielded lower accuracy classification (e.g., 87%; Spitz et al., 1996). However, biological assessments are not always feasible in large-scale epidemiological studies (Bønnelykke et al., 1989; Ooki et al., 1993; Peeters et al., 1998). This has led to the development of physical similarity questionnaires for zygosity classification.

Several studies developed zygosity questionnaires for adult twins that included questions regarding their overall similarity (e.g., "as alike as two peas in a pod") and the frequency with which they were confused by others (Cederlof et al., 1961; Nichols & Bilbro, 1966; Torgerson, 1979). Agreement between serological markers and questionnaire responses ranged from 93 to 95%. These questionnaires were subsequently adapted for young twins in which the twins and/or their parents were asked to respond to similar questions (Bønnelykke et al., 1989; Cohen et al., 1973; Ooki et al., 1990, 1993). Goldsmith (1991) has provided a thorough description of a comprehensive questionnaire developed for parents of young twins where others have borrowed elements from Goldsmith and have reported overall accuracies of near 93% (Chen et al., 1999; Spitz et al., 1996). However, some estimates of previous questionnaire accuracies were inflated by not including unclassified twin pairs in the denominator for computing the percentage correctly classified (Bønnelykke et al., 1989; Cederlof et al., 1961; Peeters et al., 1998; Price et al., 2000).

As is true with many statistically based selection procedures, classification is usually best when applied to the sample on which the classification formulae or rules were developed (Pedhazur, 1997). For this reason, cross-validation of such rules or formulae on a second sample is recommended prior to adoption. Ooki et al. (1990) performed a double cross-validation using two cohorts of different ages (i.e., developed formulae on each of two samples and then applied each formula to the other sample) and found that 95% and 67% of twins were correctly diagnosed upon cross-validation. In another study using a logistic regression equation developed in a sample of 52 pairs, 25/27 (92.6%) of a second subsample were correctly classified (Spitz et al., 1996). Although many similarity questions might seem applicable to twins regardless of sex or race, Japanese researchers have noted that questions per-

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taining to hair and eye color are of little use given the lack of variation in these characteristics within this ethnic group (Ooki et al., 1993).

Twin researchers have also varied the methods for combining questionnaire responses in order to maximize the correct classification of zygosity. The techniques used have included the use of a decision tree by Sarna et al. (1978), multiple cut-off scores by Bønnelykke et al. (1989), discriminant function analysis by Ooki et al. (1990), summation scoring by Ooki et al. (1993), and logistic regression analysis by Spitz et al. (1996). Since many of the techniques result in formulae that are sample dependent, accuracy in cross-validation studies is almost certain to be lower than in the original studies (Cohen & Cohen, 1983; Pedhazur, 1997).

The purpose of the present study was to cross-validate the classification formulae of several of these previous studies using a multiethnic sample of youth in the southeastern U.S. using the questionnaire recently described by Peeters et al. (1998). We attempted to apply the classification formulae from the original studies as closely as possible to our sample. In addition, we examined the test-retest reliability of the individual questions and of the total questionnaire, the internal consistency (Cronbach's alpha) of the total questionnaire, and the effect of receiving knowledge of the DNA results on parental responses.

Methods

The mothers of 110 same-sex twin pairs (24 MZ male and 25 MZ female European-American (EA) pairs; 3 MZ male and 6 MZ female African-American (AA) pairs; 16 DZ male and 20 DZ female EA pairs; 5 DZ male and 11 DZ female AA pairs) were selected from a cohort of 330 same-sex twin pairs. The twin pairs were recruited through announcements in local media and flyers distributed to public middle and high schools within 120 miles of the study location. The mean age was 14.6 ± 2.8 years (range = 10.8 to 25.2 years). The subjects were recent participants in a study of the heritability of the biobehavioral risk factors of cardiovascular diseases. The subsample in this study was randomly selected but was designed to represent each zygosity equally according to DNA analysis (see below). Mothers were telephoned and administered a questionnaire by a trained interviewer (Peeters et al., 1998) (Appendix, Questions 3–12). For the first question (Appendix, Question 1), the cut-off was placed between "sometimes" and "rarely". Zygosity was determined using five standard microsatellite markers (TPOX, THO1, FGA, F13A01, FES/FPS) on DNA from buccal swabs (Becker et al., 1997; Freemean et al., 1997). The likelihood of MZ using Bayes Theorem (Q = 1.8) for five concordant markers for EA and AA was 99.0% and 99.2%, respectively.

Following the initial telephone interview, 58 twin mothers were randomly selected to receive the DNA test results through the mail. Two months after the initial interview, 46 (23 MZ; 23 DZ) of these mothers were contacted and agreed to be re-interviewed using the same questionnaire after confirming that they had read the DNA results. Of the 52 mothers who did not receive the DNA results by mail, 46 (26 MZ; 20 DZ) were contacted and re-interviewed in order to assess test-retest reliability.

Three other zygosity assessments complemented the questionnaire. Two research assistants each independently examined two photographs (Olympus D-220L, 640 x 480 pixels; viewed on a VGA monitor) of the twin pairs taken under standardized conditions (one facial close up and one full body view). Twins were scored as either identical or fraternal (Table 1, Questions 13 and 14). Also, in conjunction with a laboratory visit conducted before the telephone interview, each mother was informally asked if she thought her twins were identical or fraternal (Table 1, Question 15).

Results

Table 1 provides the accuracy of determining the zygosity of MZ and DZ twin pairs separately based upon responses to the individual questions. Accuracy was computed as the percentage of the twin pairs whose zygosity was correctly identified by the responses to the question. Thus, although 100% of the siblings could accurately identify the DZ twins, the accuracy of the sibling question for DZ twins was only 88% since it could not be used for those families where the twins were the only children. No single question had greater than 80% accuracy for both MZ and DZ twin pair zygosity classification. Both the raters and the mothers were more accurate when identifying the zygosity of DZ twins (accuracy range = 94 to 95%) than they were for MZ twins (accuracy range = 71 to 80%). In addition, Table 1 shows the test-retest reliabilities for the 46 mothers who did not receive DNA test results (Pearson's r range for the ques-

Table 1

Individual Question Percent Accuracy in Determining Zygosity Classification and Test-Retest Reliability

	Accuracy MZ%	Accuracy DZ%	Reliability (Pearson's r)
Single Questions (n = 110)			
1. Mistaken for each other	97%	67%	.70
2. Long 'peas-in-a-pod'	74%	80%	.70
3. Mothers opinion	74%	87%	.57
4. 'Peas-in-a-pod'	59%	87%	.34
5. Family resemblance	71%	83%	.45
6. Parents identify	2%	100%	1.00
7. Siblings identify	4%	88%	.88
8. Teachers identify	72%	85%	.52
9. Friends identify	28%	94%	.14
10. Strangers identify	78%	77%	.55
11. Color of hair	90%	54%	.56
12. Color of eyes	93%	31%	.55
Other ratings			
13. Photo rating 1 (n = 104)	76%	94%	—
14. Photo rating 2 (n = 104)	71%	94%	—
15. Mother interview (n = 101)	80%	95%	—

Questions 3–12 were taken directly from a questionnaire developed by Peeters et al., (1998). MZ = Monozygotic n = 58; DZ = Dizygotic n = 52; Reliability n = 46.

tions = .14 to 1.00; r = .79 for the total questionnaire). The internal consistency reliability estimate (Cronbach's alpha) for the total questionnaire for the first administration was .88. An item analysis indicated this could be raised to a maximum of .90 by the omission of three questions (viz., Questions 6, 7, and 12; see Appendix).

Figure 1 gives the results of the present study and the results of several previous studies conducted by other researchers. The questions used in this study did not always exactly match those in the other studies but we attempted to replicate the original scoring schemes as closely as possible. We assumed that whatever criterion used in the original study was 100% accurate. As can be seen in Figure 1, the ability of the various questions and indices to predict zygosity was usually lower for the current study than was true in the original studies. Using the indices developed in the earlier studies, the current study had significantly (Fisher's exact test, $\alpha = 0.05$) fewer subjects correctly classified than in the original studies; 9/13 for MZ twin pairs and 7/13 for DZ twin pairs (i.e., 62% overall). Using a logistic regression equation in the current study we found that we could correctly classify 91% of both MZ and DZ twin pairs in the sample based upon the mothers' responses to 7 of the questions. As expected, this is a higher percentage correctly classified than the 86% of MZ twin pairs and the 87% of DZ twin pairs obtained from the best formulae for our sample based upon the studies reviewed in Figure 1.

The accuracy of the questions and indices did not differ when the subjects were divided into age groups based on a median split (all p's > 0.05). As shown in Figure 1, four of the indices and a single question were more accurate when classifying males than females. Furthermore, three indices and two questions more accurately classified EA than AA twin pairs.

Twelve (26%) mothers who received DNA results prior to the second telephone interview altered their opinions of their twins' zygosity from the first to the second interview (Appendix, Question 3). Ten mothers (9 MZ and 1 DZ) were concordant with the DNA results on the second interview and only 2 (1 MZ, 1 DZ) were discordant. The mother of 1 DZ twin pair considered her twins to be MZ at both interviews. Ten (22%) mothers who did not receive the DNA results also altered their opinions of their twins' zygosity between the first and second telephone interviews. Six were concordant and 4 were discordant with the DNA results on the second interview.

With respect to the internal consistency of responses within the questionnaire, we noted during the first interview that 8 mothers responded "yes" to questions 4 and 5 asking if the "children were alike as two peas in a pod" and "of only ordinary family resemblance". Seventeen mothers answered "no" to both of these questions suggesting that at least 23% of our sample were not concordant on these two questions as determined by the scoring formula.

Discussion

The use of a logistic regression formula developed on our sample resulted in 91% correct classification of both MZ and DZ twins. Only 2 of the nine earlier studies had a higher percentage of both MZ and DZ twins correctly classified (Ooki et al., 1990; Peeters et al., 1998). However, we were unable to successfully cross-validate the scoring methods used in the previous studies. Applying the formulae obtained in previous questionnaire studies to the current multiethnic sample resulted in a statistically significant smaller percentage of twins being classified correctly for 62% of the comparisons. Carter-Saltzmann and Scarr (1977) attempted to cross-validate findings of other studies, which had used responses of twins to similarity questionnaires and independent observers' ratings of photographs. These techniques did not replicate well in their multiethnic sample of 400 adolescent twin pairs: for the questionnaire, 55% of MZ and 55% of DZ twins were misclassified and for photo ratings 11% of MZ twins and 41% of DZ twins were misclassified. We would not expect as high a percentage of twin pairs to be correctly classified in cross-validation studies compared to the original studies. This is because such studies have "capitalized on chance" in two ways (Pedhazur, 1997). First, subsets of predictors (i.e., questions) were selected and, second, the weights used were calculated based upon the best fit to the data in the original samples.

Another important factor that may explain the lower proportion of twins correctly classified in our cross-validation of the original studies is the test-retest reliability. Chen et al. (1999) reported poor test-retest reliability over a 2-week period, when adolescent twin respondents had less than 80% agreement on 12 of 20 questions and their parents had less than 80% agreement on 17 of 20 questions. In order to help explain why we experienced low test-retest reliability for some of the questions, we telephoned a sample of the mothers whose answers to the questions regarding hair and/or eye color had changed between the first and second interview. In the majority of cases the mothers recalled that they had more carefully considered the questions after the first telephone interview and had changed their minds with regard to the degree of similarity of their twins' eye and/or hair color (e.g., "both twins have brown eyes but one has a lighter shade of brown"). This may reflect a problem with the telephone interview in that the respondent answers the question immediately without time for prolonged consideration. In a mailed survey such consideration might occur prior to the respondent answering the questions, thus increasing the test-retest reliability of the questions.

Question ambiguity may have also contributed to lack of high test-retest reliability. Nearly one-quarter (n = 25) of the respondents gave the same response to both the "peas-in-the-pod" question and the question asking if the twins were of "ordinary family likeness" (Appendix; Questions 4 and 5). In addition, the test-retest reliability was low for both of these questions (r = .34 and .45, respectively). As for the usefulness of the 'peas in the pod' question, our findings point in the same direction as those of a recent study by Rietveld et al. (2000) that found that this item was of minor importance in their discriminant function used for zygosity classification. Although there were some obvious problems with several of the questions in the present study, internal consistency was at a level generally considered reasonable for a questionnaire of this length (Cronbach's alpha = .88).

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Original Study	Respondents	Sample (pairs)	Criterion	Question/Index	Format	MZ Original Study	MZ Current Study	MZ p values	DZ Original Study	DZ Current Study	DZ p values	Significant Effects (p < 0.05) Race	Sex
Spitz et al., 1996	Parents	55 MZ, 24 DZ,	SSLP/	Single Questions	Mail								
		French	Dermatoglyphics	Hair color		73±6	90±4	0.030	83±8	54±7	0.020		
		8-12.5 yrs., 38 M,		Eye color		91±4	93±3	0.740	71±9	31±6	0.001	EA>AA	
		41 F		Mistaken by parents		24±6	2±2	0.001	96±4	100±0	0.320		
				Mistaken by siblings		11±4	3±2	0.160	75±9	88±4	0.180		
				Mistaken by teachers		36±6	72±6	0.001	29±9	85±5	0.001		
				Mistaken by friends		93±4	28±6	0.001	58±10	94±3	0.001		
				Mistaken by strangers		96±3	78±5	0.010	46±10	77±6	0.010		
				Of normal family resemblance		91±4	71±6	0.010	92±6	83±5	0.490		M>F
				Consider twins to be identical		47±7	74±6	0.010	100±0	87±5	0.090		
				Multiple question									
				Logistic formula based on 4 questions		95±3	95±3	1.000	88±7	44±7	0.001		
Peeters et al., 1998	Mothers	39 M MZ, 44 F MZ,	DNA/Serology	Single Questions	Telephone								
		Flemish		Consider twins to be identical		93±3 a	74±6	0.010	97±2a	87±5	0.040		
		2-31 yrs.,		Peas in a pod		93±3 a	59±6	0.001	97±2 a	87±5	0.050		
		35 M DZ, 34 F DZ		Identifiable by parents		0±0	2±2	0.420	96±2 a	100±0	0.260		
				Identifiable by siblings		3±2 a	3±2	1.000	84±5 a	88±4	0.830		
				Identifiable by teachers		70±5 a	72±6	0.850	90±4 a	85±5	0.580		
				Identifiable by friends		55±6 a	28±6	0.010	84±5 a	94±3	0.140		
				Identifiable by strangers		93±3 a	78±5	0.020	76±5 a	77±6	0.880		
				Color of hair		96±2 a	90±4	0.160	65±6 a	54±7	0.260		
				Color of eyes		96±2 a	93±3	0.450	52±6 a	31±6	0.030	EA>AA	
				Multiple question formulae									
				Unweighted Index		98±2 a	26±6	0.010	93±3 a	100±0	0.070		
				Complete Index		99±1	71±6b	0.001	96±2	73±6 b	0.001		
				Questionnaire Index		99±1	74±6 b	0.001	97±2	73±6 b	0.001		
				Short Questionnaire Index		94±3	86±5 b	0.150	97±2	87±5 b	0.040		M>F
				Similarity Index		99±1	78±5 b	0.001	97±2	83±5 b	0.010		
Chen et al., 1999	Twin Pairs	86 MZ, 19 DZ,	DNA	Regression based upon "peas in a pod"	School	99±1	53±7	0.001	89±7	83±5	0.720	EA>AA	
		Chinese		"mistaken by strangers" and									
		12-16 yrs.,		"facial appearance"									
		53 F, 52 M											

a Percentages may be inflated because "missing cases" were excluded from denominator.
b Percentages may be inflated because original article did not give actual variable weights so current study used optimal weights.

Figure 1
Comparisons of Questionnaire Accuracy with Previous Studies

Original Study	Respondents	Sample (pairs)	Criterion	Question/Index	Format	Accuracy (% ± Std. Error) MZ Original Study	MZ Current Study	MZ p values	DZ Original Study	DZ Current Study	DZ p values	Significant Effects (p < 0.05) Race	Sex
Cederlöf et al., 1961	Twin Pairs	88 MZ, 112 DZ, Swedish 35-75 yrs., sex--na	Serology	"Peas in a pod" & "mixed up" by parents, siblings, teachers	Mail	82±4	74±6	0.300	93±2	63±7	0.001	EA>AA	
Sarna et al., 1978	Twin Pairs	56 MZ, 48 DZ, Finnish 20-69 yrs., sex--na	Serology	Decision tree based upon: "two peas in a pod" and "Could parents, siblings, schoolmates, strangers tell twins apart?"	Mail	95±3	64±6	0.001	88±5	85±5	0.780		M>F
Bønnelykke et al., 1989	Mothers	78 MZ, 47 DZ, Danish 6 mos.-6.5 yrs, sex--na	Serology	Decision tree based upon: Haircolor/Eye color ordinary family resemblance peas in a pod and mixed up by family or friends	Mail	96±2	64±6	0.001	83±5	81±5	0.800		M>F
Ooki et al., 1990	Twin Pairs	165 MZ, 24 DZ, Japanese 12-16, sex--na	Genetic Markers	Discriminant function analysis based on "peas in a pod" and "mixed up" by parents, teachers, others	Self-Admin.?	92±2	97±2	0.360	88±7	58±7	0.020	EA>AA	
	Twin Pairs	71 MZ, 22 DZ, Japanese 50-77 yrs., sex--na	Genetic Markers	Discriminant function analysis based on "peas in a pod" and "mixed up" by parents, teachers, others	Self-Admin.?	94±3	78±5	0.010	91±6	69±6	0.080		
Ooki et al., 1993	Mothers	61 MZ, 13 DZ, Japanese High School, sex--na	Genetic Markers	Total score based upon "peas in a pod" and "mixed up" by parents, teachers, others	Self-Admin.?	98±2	55±7	0.001	77±12	92±4	0.140		

Figure 1 continued

Comparisons of Questionnaire Accuracy with Previous Studies

The southeastern U.S. represents a population for which twin zygosity questionnaire assessment has not been previously reported. For three of the indices and two individual questions concerning eye color, EA twins were more likely to be diagnosed correctly than AA twins. The reasons for this are probably related to the lack of variation in hair and eye color in AAs, which parallels the concerns of Ooki et al. (1993) in Japanese twins. Males were also more accurately assessed by three of the indices. This may be due to the tendency of females, in particular, to alter their appearance with cosmetics or hair styling. For most questions and indices, however, differences by gender and ethnicity were not statistically significant.

In the subsample that received results of the DNA test by mail, 12 mothers (26%) changed their opinion when asked directly about the zygosity of their twins (Question 3), which suggests that mother's opinion can easily be changed by DNA evidence. Ten of 12 (9 MZ, 1 DZ) changed their responses to be in concordance with the DNA results. Seven of the 9 MZ mothers indicated that their original and incorrect designation of DZ was primarily due to "the overall similarity or dissimilarity of their physical features" (Question 2b: see Appendix). Of the 22 mothers whose opinions on the first interview were discordant with the DNA testing results, 15 were mothers of MZ twins. This preference of labeling a twin as dizygotic is in agreement with other studies (Rietveld et al., 2000). The main reason seems to be that parents tend to emphasize relatively minor phenotypic or behavioral differences as evidence of DZ (Machin, 1996), as indicated by responses to Question 2b (see appendix). When multiple raters agree on a classification, the twins are less likely to be referred for a biologic test than if there were a disagreement between raters. In our study when there was 100% agreement between the two raters and the mother's initial laboratory assessment on a classification of dizygosity, 6 of 47 times (12.7%) the assessment was wrong (i.e., the twins were actually MZ). When there was 100% agreement on a classification of monozygosity, 1 of 29 times (2%) the classification was wrong. Thus, selection for biological testing only in cases where classification is "uncertain" may still lead to some misclassifications.

There were a number of methodological differences between the enumerated studies and the present study that may partially account for poor cross-validation including the questionnaire respondents, age of the twins, selection of questions, and the format of the questionnaire. Having the twins rather than their mothers answer the questionnaire might have altered the results. However, Ooki (1990) found that the responses of mothers to a zygosity questionnaire could be used to classify their adolescent twins at least as well as responses of the twins' themselves. Although the ages of twin pairs might alter the ability of questionnaire responses to classify zygosity, all of the studies reviewed (with the exception of Cederlof et al., 1961) had quite high levels of correct zygosity classification despite the wide age range of the twins involved. As discussed above, the questions used in a zygosity classification questionnaire and the format of such a questionnaire influence the ability to correctly classify twin pairs and should be carefully considered when designing such a questionnaire.

In conclusion, similar to Carter-Saltzmann & Scarr (1977), our findings suggest that if biological based zygosity classification is not feasible in all subjects, rather than using classification indices based on other studies, an optimal classification scheme can be achieved by using a zygosity questionnaire of which the reliability and criterion related validity of the questions is established in a random subsample of the same twin cohort (e.g., Price et al., 2000).

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Appendix

Questionnaire

1. Please answer frequently, sometimes, rarely, or never to the following question. "Are your twins mistaken for each other by people who know them?" ___Frequently ___Sometimes ___Rarely ___Never.

2a. Non-identical twins are no more alike than ordinary brothers or sisters. On the other hand, identical twins have such strong physical likeness to each other in height, coloring, facial features, etc., that people often mistake one for the other or say they are "as alike as two peas in a pod." Having heard these statements, do you think your twins are physically identical twins or non-identical twins? ___Physically identical ___Non-identical

2b. Based on your last answer, please rank the following items in the order that they influenced your answer. The most influential factor should be ranked number one, the next number 2, with the least influential factor as number three. A) I just know. B) What the physician told me about the placenta C) The overall similarity or dissimilarity of their physical features.

3. Do you think your twins are identical or fraternal?	___Identical	___Fraternal
Are you sure that your twins are (*above answer*)	___Yes	___No
4. Are the children as alike as two peas in a pod?	___Yes	___No
5. Are the children of only ordinary family resemblance?	___Yes	___No
6. Who can identify each twin — parents?	___Yes	___No
7. — brothers and sisters?	___Yes	___No
8. — teachers?	___Yes	___No
9. — friends?	___Yes	___No
10. — strangers?	___Yes	___No
11. Are there differences in your twins' hair colors?	___Yes	___No
12. Are there differences in your twins' eye colors?	___Yes	___No