

# Why Zygosity of Multiple Births is not Always Obvious: An Examination of Zygosity Testing Requests From Twins or Their Parents

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This paper examines why parents of twins or adult twins themselves request zygosity testing. Of 405 multiples including 8 sets of triplets, the majority (93%) were monozygotic. Age of testing ranged from 0 days to 73 years. About 50% of requests came from parents or twins who were curious about, or expressed a need to be certain of, their zygosity. Other reasons included health concerns (current or future), other twins in the family, and misinformation about zygosity, frequently because of the erroneous assumption that all dichorionic twins are dizygotic. Parents of monozygotic twins may expect their twins to be 'identical' and believe their twins to be dizygotic because of minor phenotypic differences between them. Dizygotic twins like other siblings may share a phenotypic resemblance. Health professionals should be aware that zygosity of multiples may not always be obvious to parents and that accurate knowledge of zygosity may be justified.

Twins are not uncommon in the population. The incidence of spontaneous twins is about 1:80 deliveries, and 1:40 individuals are one of a twin pair, although the incidence in different ethnic groups ranges from 1 in 35 in the U.S. to 1 in 120 in Japan (Ventura et al., 2001). The incidence of multiple births in the population is increasing. Women are delaying having children until a later age when fecundity is sub-optimal and may require fertility drugs or *in vitro* fertilization (IVF) to achieve a pregnancy. Spontaneous dizygotic (DZ) twins increase slightly with the age of the mother. The increased incidence results from increasing success and improved access to assisted reproductive technologies (ART), as well as increasing maternal age (Jones, 2003). Studies in both Denmark and the U.S. have shown an increase in the incidence of twins in both populations. The Danish data showed an increase from 10.2 per 1000 births in 1980 to 16.7/1000 births in 1994 (Westergaard et al., 1997). In the U.S. the incidence increased from

18.9/1000 births in 1980 to 28.9/1000 births in 1999 (Ventura et al., 2001). The incidence of triplets has increased in Denmark from 11.1/10,000 births in 1980 to 73.2/10,000 births in 1999, while in the U.S. the incidence has increased from 29.1/10,000 births in 1971 to 116.2/10,000 in 1997 (Martin et al., 1997).

One third of all twins are monozygotic (MZ), developing from a single fertilized ovum, while two thirds are DZ. Unlike DZ twinning, the rate of MZ twinning is independent of population differences and maternal age effect, and there has been little change in MZ twinning rates (Machin, 1999a). While *in vitro* fertilization mostly results in DZ twinning and trizygotic triplets, there has been a slight increase in MZ twinning rates as well, particularly following assisted hatching techniques (Schieve et al., 2000). An examination of higher multiples showed that monozygosity is more common in spontaneous triplets than in IVF triplets. In a study of 15 spontaneous triplets only 2 were trizygotic (Machin & Bamforth, 1996).

Multiple births have a higher morbidity and mortality rate than singleton pregnancies, largely as a result of prematurity but also because of unique complications in MZ twins (Machin et al., 1995; Machin, 1999b). As part of an initiative to identify and diminish the complications that may occur in a multiple pregnancy, zygosity testing on placental samples from multiple births is routinely undertaken at the University of Alberta Hospital. Because of an increasing number of zygosity requests from parents and adult twins, testing was later extended on a cost-recovery basis to these individuals.

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In this paper we examine the various reasons why parents of twins or twins request zygosity testing and why knowledge of zygosity is important to them. Our objective is to raise awareness of the importance of giving accurate information about zygosity to twins and parents of twins. There is generally a misconception among pediatricians and obstetricians that zygosity will immediately be obvious to parents of twins — that is, DZ twins will easily be distinguishable from MZ twins who are identical — however, in practice, the term ‘identical’ twinning implies that MZ twins should be identical. In the authors’ experience, parents and people close to the family have no difficulty in telling their MZ twins apart although individuals outside the close family may think they are ‘identical’ (Bamforth & Machin, 1994). There are several reasons why MZ twins may differ phenotypically. These can be divided into genetic and acquired factors. Genetic differences may include postzygotic chromosomal anomalies and discordance for congenital anomalies, difference in severity of inherited disease, for example, Fragile X mental retardation syndrome, and tuberous sclerosis (Machin, 1996). There may be differences in X-chromosome inactivation patterns in female MZ twins (Bamforth et al., 1996; Goodship et al., 1996; Jorgensen et al., 1992; Lupski et al., 1991), differences in imprinting, for example, discordance for Beckwith–Wiedemann syndrome (Leonard et al., 1996), and mirror-imaging (Sperber et al., 1994). While discordance for anomalies may be obvious, it is reasonable to expect that minor differences in gene imprinting, including X-chromosome inactivation patterns, might explain some of the reasons why MZ twins are not identical. The most likely candidates would be growth factors, genes coding for cell adhesion molecules and those influencing cell differentiation (Bamforth et al., 2003). These could contribute to birthweight discordance in MZ twins, as well as to complications, for example, the development of placental vessel anastomoses implicated in twin–twin transfusion syndrome.

Discordance for birthweight may also reflect the intrauterine environment with unequal sharing of a monochorionic (MC) placenta. Severe growth discordance (> 20%) is commoner in MZ MC twins and DZ twins than in MZ dichorionic (DC) twins (Machin et al., 1995). Other acquired differences may result from compression of the two fetuses and include craniosynostosis and head shape. After birth, illness in one twin may result in phenotypic difference in twins, for example, head molding and size in premature twins. DZ twins, like other siblings, share about 50% of their DNA and it is therefore not surprising that there may be a striking phenotypic resemblance between them, as there may be for other siblings at the same age.

Placentation may help in discerning zygosity. DZ twins account for 70% of twins. Of these 50% will be of unlike gender. MZ twins account for 30% of all

twins and two thirds will be MC. With rare exceptions, zygosity can be ascertained without resort to further testing in 55% of twins who are either unlike gender or MC. However, the placenta may not always be available for detailed examination and errors in assigning mono- or dichorionicity based on the analysis of membranes may occur. Zygosity is unknown for all like-sex DC twins accounting for 45% of all twins.

## Materials and Methods

Zygosity testing has been routinely undertaken on placental samples from like-sex multiple births since 1989 at the University of Alberta Hospital, Edmonton. After DNA extraction, DNA is analyzed using microsatellite DNA, with 99.99% accuracy in assigning zygosity (Bamforth, 1999). We also offer zygosity testing for parents and twins interested in knowing their zygosity on a cost-recovery basis. Since 2002 we have been offering testing for nonmedical purposes using buccal swab samples, thus avoiding the need for collecting a blood sample. Buccal swabs are collected by twins or their parents. DNA extracted from buccal swab samples is analyzed by DNA amplification of seven polymorphic markers (PM/DQA, Applied Biosystems) and because of its lower accuracy (99.2%), individuals are informed that this is not sufficiently accurate for medical purposes.

Between 1989 and 2003 there have been 405 requests for zygosity testing from parents or twins themselves. Although there has been no formal request for information, parents frequently volunteer information about their twins and in this paper we examine the various reasons why zygosity testing has been requested. Information collected included reasons for requesting testing, any discordance (e.g., mirror-imaging, size, anomalies), other twins in the family, placentation (either a histological report or the presence of one or two placentas) and twins’ or parents’ perception of zygosity either from their own observations or what they had been told.

Reasons for requesting zygosity, where available, were classified as follows, based on written information which accompanied the requests:

1. Need to know — this category included the following statements, or similar:
  - ‘Important to know’ or ‘I want to know’
  - ‘Important for my twins to know their zygosity’
  - ‘I need to be certain’.
2. Curiosity — this category included the following statements, or similar:
  - ‘Curiosity’
  - ‘We think identical, but differences’ or ‘We think fraternal, but alike to others’
  - ‘Always being asked’, ‘Sick of not knowing’.
3. Health reasons — this category included:
  - twins discordant for a condition

**Table 1**  
Age, Gender and Zygosity Distribution of Twin-Zygosity Requests Expressed as Percentage of Total Requests

Age group (years)	No.	Female %	Male %	Gender unknown %	DZ %	MZ %	Result N/A %
0–1	125	49.6	47.2	3.2	8.0	89.6	2.4
2–4	128	55.5	44.5	0	5.5	94.5	0
5–9	62	53.2	43.6	3.2	3.2	92.0	4.8
10–17	25	48.0	52.0	0	8.0	92.0	0
All children	340	52.3	45.9	1.8	6.2	92.0	1.8
Adults	36	83.3	16.7	0	0	100	0
N/A	21	52.4	47.6	0	4.8	90.4	4.8
Total	397	55.2	43.3	1.5	5.5	92.7	1.8

concern for future health including transplantation present health concerns for twins or family.

4. Misinformation — this category included: dichorionic twins called ‘fraternal’ other reasons.
5. Familial twinning — this category included: ‘twins in the family’ as the sole reason for the request.

Each category was stratified by age and subject to statistical analysis using the *t* test.

**Results**

There were 397 twins and 8 triplets included in the zygosity requests. Thirty-six (9%) requests came from adult twins while 369 (91%) requests came from parents. Tissues examined for DNA polymorphisms included cord blood, (5) archived paraffin sections of placenta (69), blood samples (316) and buccal swabs (15). Three hundred and eighty-five samples were analyzed by DNA microsatellites and 20 (15 buccal swabs, 5 archived placental samples) by PM/DQA kit (Applied Biosystems). In a minority of cases, results for zygosity testing were not obtained (9/405). All these were paraffin blocks of archived placental samples in which DNA was poorly preserved. The

median age of testing was 3 years, range 0 days to 73 years (Table 1).

Two thirds of multiples were less than 4 years of age at the time of the request. Of the twins in the 0–17 years age group there were approximately equal numbers of females and males. However, of the 36 adult twins who requested testing, there was a predominance (83%) of female twins — although this was not statistically significant (*t* test *p* > 0.5). Overall, 93% of all twins tested were MZ. The percentage of twins tested who were MZ increased with age, increasing from 90% in the 0–1 year age group to 100% in the adult twins. Of the 8 sets of triplets, 5 were all-female and 1 was all-male. Median age of testing was 1 year, range 2 months to 6 years. Three were MZ; the remaining sets were MZ/DZ. There were no trizygotic (TZ) triplets in this group.

The reasons for zygosity testing were given in 184 (45%) of the requests and are shown in Table 2. The most common requests in the 0–17 years age group were because of curiosity (54/156, 35%) and ‘need to know’ (47/156, 30%) but among the adults the commonest reasons were health concerns (9/28, 32%) and curiosity (8/28, 29%).

Information on chorionicity was included in 147 cases (Table 3). In 62 cases, predominantly where chorion was analyzed, placental histology was available. In the remaining 85 cases, chorionicity was

**Table 2**  
Categorization of Zygosity-Testing Requests Expressed as Percentage According to Age Group

Age at testing (years)	No.	Need to know %	Curiosity %	Health concerns %	Misinformation %	Familial twinning %
0–4	112	30.4	34.8	18.8	10.7	5.3
5–17	44	29.5	34.1	13.6	13.6	9.2
All children	156	30.1	34.6	17.3	11.6	6.4
Adults	28	21.4	28.6	32.1	0	17.9
<i>t</i> test: <i>p</i> adults vs. children		NS	NS	NS	NS	NS
Total	184	28.8	33.7	19.6	9.8	8.1

Note: NS = not significant

**Table 3**  
Chorionicity and Zygosity Results Expressed as Percentage

Chorionicity	No.	Zygosity		
		MZ %	DZ %	No result %
DC by histology	44	72.8	13.6	13.6
DC—two placentas	62	100	0	0
All DC placentas	106	88.8	5.6	5.6
MC by histology	18	66.6	16.7	16.7
MC—one placenta	23	100	0	0
All MC placentas	41	85.4	7.3	7.3
Total	147	87.8	6.1	6.1

Note: DC = dichorionic placenta, MC = monochorionic placenta

inferred from the presence of one or two placentas. It is acknowledged that one placenta may include a fused DC placenta. As expected, the majority (106/147) of twins were dichorionic (72%). Of the DC placentas, 94 (89%) were MZ. Of the MC placentas, 35/41 (85%) were MZ. However, 3 histologically MC placentas were from DZ twins.

Some twins volunteered information about the occurrence of other twins in the family, probably because of our interest in this topic. In 125 cases there were other twins in the family — vague statements about twins in the family were discounted. In 35 (30%) there were more than one set of identical twins, and in 21 (17%) there were more than two sets of multiples in the family. Only 15 parents or twins (8%) indicated this as the reason for requesting zygosity. Misinformation about zygosity accounted for 18 requests (12%), all by parents of multiples.

## Discussion

This paper examines zygosity-testing results on 405 multiples (397 twins and 8 triplets) where the request was made by adult twins (9%) or parents of multiples (91%). The study naturally reflects a highly self-selected population of multiples. It excludes those parents or twins who feel certain of their zygosity or for whom zygosity is not an issue. Most zygosity requests (93%) came from parents of MZ twins or adult MZ twins. The MZ twins in this study may represent those who are less alike than other MZ twins. The percentage of MZ twins increased with age of testing. Among the 36 adult twin pairs, all were MZ.

Most requests came from parents of multiples under the age of 4 years when zygosity may be more difficult to decide from appearance alone. Among the children (0–17 years of age) tested, there was an equal distribution of females and males (1.14:1). For the adults, the female/male ratio was 5.8:1. It is impossible to know whether female twins are more curious about their zygosity or whether there are greater phenotypic differences in adult female MZ twins than in male MZ twins. One could speculate that differences in X-chromosome activation patterns could

contribute to a greater phenotypic difference in female twins.

The reasons for requesting zygosity testing differed in adults and children. Curiosity was the main reason for requests in children and health the main reason in adults. The comparison of adult and child requests did not reach statistical significance, possibly because of the small number of adults in the study.

Health issues were given as the major reason for zygosity testing in 9/28 (32%) of adults, 21/112 (19%) of the 0–4 year age group and 6/44 (14%) of the 5–17 years age group. However, the health reasons cited differed in children and adults. In children, reasons included current health concerns, for example, allergies; size differences (9/27); discordance for an anomaly (9/27); future health concerns, including transplantation, (6/27); and family planning, including recurrence risk of twins, (3/27). Two parents requested testing because another child in the family had a significant health problem. The current-health-concern group included four sets of triplets, two IVF multiples (one a triplet pregnancy) and two pregnancies resulting in stillbirth (one of triplets) or neonatal death from multiple congenital anomalies (one of MC twins). In the case of 6 of the 9 adults, all MZ, there was discordance for adult-onset disease — 4 cases of cancer in a co-twin and 2 cases of renal disease in a co-twin. The remaining twins were discordant for congenital anomalies. With the increasing recognition of the genetic contribution to adult-onset diseases, for example, cancer or type 2 diabetes, we might expect to see more zygosity requests from adult twins in the future.

In the need-to-know group, there was little elaboration on the reasons, although 3 parents mentioned that it might influence educational choices for twins and another that her twins had the right to know their zygosity. In the curiosity group, there were a variety of reasons for requesting testing. In this group monozygosity was confirmed in 17/17 twins whose parents thought them to be MZ and of 15 twins thought by their parents to be DZ, 14/15 were MZ. This confirms our experience that lack of knowledge about phenotypic differences in MZ twins, for example, discordance or mirror-imaging, leads to uncertainty about their zygosity (Bamforth & Machin, 1994).

Some requests included information about other multiples in the family. It is well known that there is a genetic contribution to twinning, particularly DZ twinning (Eriksson, 1990; Lichenstein at al., 1996). In 15 cases the only reason given for requesting zygosity was that there were other twins in the family. While this may reflect a genetic predisposition it should also be remembered that twinning is not uncommon and this may be reflected in a larger pedigree. However, in 21 (17%) cases there were more than two sets of multiples in the family; these cases are more likely to indicate familial twinning.

Misinformation about zygosity accounted for only a small number of requests in children (18/156, 12%) and no adults. Two twin pairs were labeled DZ because of marked differences in birthweight. Fifteen pairs were MZ DC twins and labeled 'fraternal' at birth. Placentation may not always be reliable. Among the MC twins determined by histology, all would be expected to be MZ, although 3/41 (7%) were DZ. While error cannot be excluded, there are rare reports of DZ twins arising from monochorionic placentation (Langlois et al., 1994; Souter et al., 2003) and this may be a more common phenomenon than previously recognized.

Health professionals should be able to provide accurate information about zygosity where this is requested and also understand that health issues related to twinning, either present or future, are a concern for twins or their parents. The misinformation, curiosity and need-to-know categories of requests may reflect a lack of information about zygosity. Zygosity testing should be available to twins and their parents and knowing zygosity may be reassuring.

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