B. REVERSADE

TWO BY TWO

Could genes explain the remarkable rate of identical twins born in some remote villages around the world? **David Cyranoski** investigates a long-standing biological curiosity.

In December 2008, Bruno Reversade travelled to India in pursuit of some spit. The journey took him first to the north-eastern city of Allahabad, and then a further 10 kilometres to Mohammad Pur Umri, a farming village enclosed by mud walls. There he asked some of the 2,000 or so residents to deposit samples of their saliva in a cup specially designed for the purpose. Fifty-five of them complied.

This is not the first time that these villagers have sacrificed their body fluids for science. Mohammad Pur Umri has become somewhat famous, not for the milk or mustard that provides the villagers with their livelihood, but for its prolific production of identical — monozygotic — twins. Globally, only 1 in every 250 to 300 births are identical twins. In Umri, roughly one in ten is of this type, births that the villagers — including the twin village leaders — call "gifts from God".

Reversade is looking for genes that might be responsible for this gift. "Every 50 seconds a pair of natural clones is born. It's more frequent than some of the most frequent genetic diseases," says Reversade, a developmental biologist at Singapore's Institute of Medical Biology. "It can't be random." Many scientists disagree, arguing that chance could fully account for this cluster of cases and for every twin birth besides. Aside from genes and chance, theories abound for what causes a fertilized egg to produce a pair of monozygotic twins. None is well accepted. Nor do scientists understand how entities that are apparently genetically identical can come to have such different personalities and disease susceptibilities. Environmental factors explain some, but not all, of these differences.

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Over the past few years, scientists have been using new genetic and cell-biology techniques to attack these questions. Reversade is gathering samples from three 'twin towns', and using genomic analyses that, he hopes, will point to a common molecular pathway involved in twinning. Embryologists and obstetricians are looking for clues in assisted reproduction (see 'Making twins'), which is known to promote monozygotic twinning as well as high rates of dizygotic twins, which result from the transfer of multiple embryos. Theories on how identical twins come to differ are also being overhauled, with some studies even suggesting that genetically identical twins may never, even at the earliest stage of development, have been genetically identical. "This research just hasn't been part of people's thinking," says Judith Hall, a prominent twinning researcher at the University of British Columbia in Vancouver.

Reversade became interested in monozygotic twinning after cutting frog embryos into halves and watching through a microscope as they developed into identical embryos. "Pure awe," he says. His goal is to understand why cells that are acting together to form an embryo split off and start building a whole new organism, something they can do early, when the embryo is just a few cells big, or as late as two weeks into development. Conjoined twins can result if the embryo splits too late or incompletely. Reversade says that twinning offers the best way to study 'regulative development' — the interaction between cells that informs each one when to follow the pack and when to act alone. He moved to Singapore in February 2008 after landing the government's first A*STAR Investigatorship, a US$500,000

P. ADHIKARY/EPA/CORBIS

B. MERRIMAN

Bruno Reversade (left) is collecting DNA from various 'twin towns'.

per year grant modelled on the Howard Hughes Medical Institute awards, having convinced the funders of the feasibility and importance of finding a twinning gene or genes.

Twinning is often assumed to run in families, and in the case of dizygotic twins, caused by the release and then fertilization of two eggs, scientists agree that this is the case. So far though, researchers have only found genes that are weakly correlated with dizygotic twinning. The picture for monozygotic twins is even less definitive. In large-scale studies, family members of the mothers of monozygotic twins do not seem more likely to have monozygotic twins themselves[1]. But some families and some communities do produce identical twins in numbers that seem to defy this interpretation. Reversade is visiting them one by one.

Two years ago he travelled to Jordan to collect saliva samples from members of a family with 15 pairs of monozygotic twins. The family tree fits a pattern in which a dominant gene — one that would cause monozygotic twinning even if only one copy is present — is on one of the 22 autosomal chromosomes. But to make the hereditary pattern work, the gene must have 'variable penetrance', such that some women would not bear monozygotic twins even though they carried a copy of the gene. "Variable penetrance is of course a 'black box'," says Reversade. "Why don't we see more twins?" One reason, he suggests, is that some twins 'vanish' — meaning that at least one of the two dies — during pregnancy.

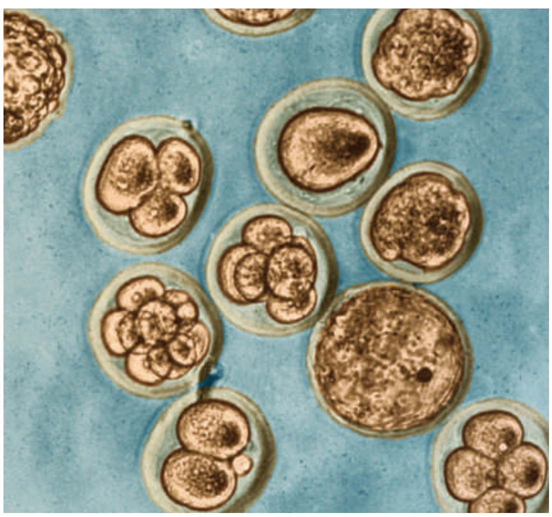
According to a widely cited estimate by Charles Boklage, a behavioural and developmental biologist at East Caroline University in Greenville, North Carolina, at least 12% of natural conceptions will produce twin embryos. Both twins come to term in only 2% of those pregnancies. A singleton is born around 12% of the time, and in the vast majority of cases both embryos are lost, often without the pregnancy ever being noticed[2,3]. Some theories suggest that the twin conceptions that survive do so because of a healthy system that is able to support two embryos through implantation and pregnancy.

"Humans do it any old time and can easily miss the right time." — Judith Hall

Founder effect

In Jordan, Reversade used genetic tests to search for shared patterns of single-letter variations in the genomes of the twins that might point to a twinning gene. He found one candidate region, on chromosome four. One of the genes in the region has some promising characteristics: it is conserved through vertebrate evolution, it codes for a protein that is expressed in the blastocyst stage of mice embryos, and its activity drops once the cells that make it have differentiated. Reversade thinks that mutations in the gene or other genes working in the same signalling pathway might have been present in the founders of each twin town and then spread through the population. He says he won't publish the work "until I have the full story, namely the gene, and a possible mechanism".

That story took on new twists during Reversade's visit to India. "The plot thickens," Reversade wrote in an e-mail after returning from Mohammad Pur Umri. "The last pair of monozygotic twins was born 3 weeks ago. There have been close to 55 pairs over the past 30 years. Not all have survived. One mother has had two pairs of monozygotic twins. It is absolutely astounding." Villagers there told him of three sets of twins produced by buffalo that share a pond the village uses for its water supply. Reversade took samples of the water to check for any substances that could affect reproduction. The beginning of twinning in the village coincides with the establishment of a nearby air force base some 40 years ago, but he could not find any evidence of obvious environmental pollutants. Still, some villagers are convinced there is something in the soil causing the twinning.



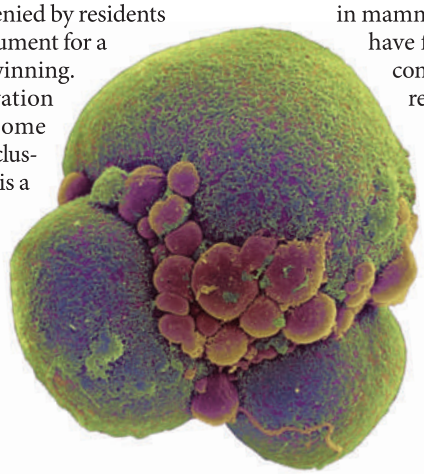
M. JOHNSON/WELLCOME IMAGES

Human embryos can split and become twins early or late in development.

At least one related gene hunt has been unsuccessful. In 2004, scientists from the Centre for Cellular and Molecular Biology in Hyderabad collected blood samples from the Indian village to look for genes associated with twinning and appointed a sociologist to survey food intake and social habits. "We have not been able to conclude anything significant," says Lalji Singh, the centre's director. The group did not bother publishing the results.

Reversade hopes that newer technologies — he plans to use next-generation genetic sequencing machines on the candidate regions in all his subjects — will give him the necessary sensitivity to find genes, but he has a long way to go to convince his peers. Edison Liu, director of the Genome Institute of Singapore, says that inbreeding, which is suspected in each of these villages (although denied by residents of Umri) supports the argument for a genetic contribution to twinning. But, he says, "the observation should be viewed with some scepticism as geographical clustering of rare events often is a statistical fluke".

G. MOSCOSO/SPL



Cells in the early embryo may lose their ability to stick together.

Reversade's next stop, probably this summer, will be the village of Linha São Pedro in Brazil, a town predominantly of blond-haired, blue-eyed people founded by German immigrants. In the 1990s, 10% of the births there were twins, and almost half of those were monozygotic. In a book published last year, *Mengele: the Angel of Death in South America*, Argentine journalist Jorge Camarasa argues that Nazi doctor Joseph Mengele was responsible, using techniques he devised to rebuild a master race. The Mengele argument hasn't sold well among scientists and Reversade hopes to prove it wrong once and for all. "That explanation makes it a perfect plot to solve," he says.

Humans are one of the most proficient mammalian species at multiple monozygotic births. Armadillos are better. The nine-banded armadillo (*Dasypus novemcinctus*) produces identical quadruplets with every litter from embryos that have split, and then split again. Importantly for Reversade, they also show that multiple births can have a genetic basis in mammals. Evolution seems to have favoured the genes that contribute to the armadillo's reproductive strategy. Creating multiple embryos from each egg may get around physical constraints imposed by the shape of the armadillo uterus.

It is not clear that reproduction in humans and armadillos would employ the same genes or have arisen for the same evolutionary reasons. Most scientists think of twinning in humans not as an evolutionary advantage, but as a breakdown of normal function in a female body evolved to carry only one fetus at a time. Some, taking into consideration the 2–3 fold increased risks of congenital anomalies in babies born as monozygotic twins[4], describe it in terms of risk factors, as they would a disease. "Most people accept that twinning is a failure, not a desired outcome," says Dianna Payne, an embryologist and visiting researcher at the Mio Fertility Clinic in Yonago, Japan.

Why the system goes awry is not clear. One theory of twinning holds that subtle differences force cells in early embryos to repel one another and establish two separate cell masses. Reversade, hesitantly, speculates that his mutated candidate gene might deprive cells in the embryo of their ability to adhere tightly to each other, resulting in a split.

Hall favours a theory based on the timing of fertilization to explain why humans twin so often. Most mammals recognize the oestrus cycle so that they mate at "the right time", she says, when eggs have been freshly ovulated. "Humans do it any old time and can easily miss the right time." She suggests that the outer shell, called the zona pellucida, of an old egg might be more likely to fracture and split the blastocyst in two when it later hatches out[4]. In this scenario, twinning could just be a side-effect of an otherwise successful human reproductive strategy.

Normal genetics

Isaac Blickstein of the Hadassah-Hebrew University School of Medicine in Jerusalem and Louis Keith of Northwestern University in Chicago, Illinois, think that there could be a genetic component to monozygotic twinning, although not one considered mutant or abnormal. They argue that human twinning is the result of "a subpopulation of primary oocytes with an inborn and, as yet, unspecified propensity" for splitting[5].

There is one thing that all scientists agree on: assisted reproduction causes higher rates of monozygotic twinning — 2 to 12 times higher, according to a review published last year[6]. Ovarian stimulation with drugs, and embryos

MAKING TWINS

The clonal propagation of human twins might sound like the setting for a science fiction novel, but it is already being tested for some types of fertility treatment.

Karl Illmensee, laboratory director at the Genesis Fertility Center in Patras, Greece, thawed and then manually cleaved human embryos at different stages of development then cultured the resulting sections *in vitro*. He found that splitting high-quality embryos at the 6–8 cell stage (pictured) is the most promising way to create viable twin embryos. He split 12 embryos that had been fertilized *in vitro* and developed to this stage, and found that 16 of the resulting 24 embryos developed into healthy, mature (morula or blastocyst) embryos[10].

These embryos were at the stage at which they could be transferred to the uterus. But Illmensee experimented with 'triploid' embryos, which have three, instead of two, sets of chromosomes. These embryos don't usually survive to term and are discarded during *in vitro* fertilization (IVF) procedures.

Illmensee theorizes that the technique would give couples who are low on embryos after IVF a greater chance of becoming pregnant. But would their offspring be healthy? Various animals, including mice, sheep, cattle, goats, pigs and horses have produced

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twins after embryo splitting. But efforts by scientists to make genetically identical primates by this technique, for use as a disease model, have resulted in multiple pregnancies but only one live birth, and no twins[11,12]. Illmensee points to only one attempt to create human twins in a laboratory, in which the split embryos stopped developing after just a few cell divisions[13].

Illmensee left the scientific mainstream after some of his experiments were called into question in the early 1980s, including some in which he claimed to clone mice. He says he is being careful to avoid potential ethical objections. He quotes the American Society for Reproductive Medicine's ethics committee report on twinning: "splitting one embryo into two or more embryos could serve the needs of infertile couples in several ways. As long as a couple is fully informed of the risk of such an outcome, there would appear to be no major ethical objection." **D.C.**

B. LAVIES/NATIONAL GEOGRAPHIC/GETTY IMAGES

Nine-banded armadillos consistently give birth to genetically identical quadruplets.

spending an extended period in culture before being transferred are most consistently linked with twinning events. The culture conditions could act directly on the embryo, encouraging it to split, or could harden the zona pellucida so that the embryo is snipped in two as it hatches.

Perhaps the closest researchers have come to watching human twinning is in time-lapse images collected every two minutes during the development of embryos that had been created by *in vitro* fertilization, frozen and then thawed after they were designated as surplus. Payne's team at the Mio Fertility Clinic was surprised to find that 25 of 26 blastocoels, fluid-filled cavities that support the compacted clump of cells that will become the fetus, collapsed at least once. The more frequent and more dramatic the collapse, the less likely the embryos were to survive. Most strikingly, the clump of cells inside two of the collapsed blastocoels split and the two fragments developed as if they were on the path to becoming two separate embryos in different parts of the egg. "The cells are quite sticky at that point and when the blastocoel reinflates itself, some get stuck on the other side," says Payne. She thinks that the culture system, which does not perfectly mimic the uterine environment, might bring about the collapse by causing some cells to die or by weakening cell junctions in the egg's membrane. Such mechanisms could also be taking place in naturally conceived embryos if they were triggered by faulty genes, says Payne. "But we'll never get a camera in there to see it."

"Most people accept that twinning is a failure, not a desired outcome."
— Dianna Payne

Special cameras aren't needed to document another aspect of identical twinning: that the twins differ in appearance, personality and in their propensity to develop disease. "After 50 years of epidemiological work, we cannot answer why there is such divergence in multiple sclerosis, schizophrenia, or type I diabetes between twins," says Arturas Petronis, who has been studying twins at the University of Toronto in Ontario.

Last year, Jan Dumanski at the University of Alabama in Birmingham offered a surprising possible answer: that identical twins are not so genetically identical after all. Her group compared 19 pairs of monozygotic twins and found that the individuals within a pair have segments of DNA that are duplicated or deleted[7]. These regions might help to identify the causes of some of the disease discrepancies within the pair.

Other explanations for twin differences might be found outside the genetic sequence. Manel Esteller at the Bellvitge Institute for Biomedical Research in Barcelona, Spain, looked for differences in patterns of histone acetylation and methylation — 'epigenetic' marks that commonly control gene activity — in monozygotic twins ranging in age from 3 to 74. The younger twins had similar epigenetic marks — but those patterns diverged with age, or as they were exposed to different environmental factors[8]. "When one twin starts smoking, taking drugs or moves somewhere with more air pollution — even for only a year — their epigenetic profile can diverge sharply," says Esteller. "It is very dynamic." Petronis, however, thinks that spontaneous, random epigenetic changes are likely to contribute more than those triggered by the environment.

The epigenetic differences might even start accumulating from day one of development. Researchers at the University of Cambridge, UK, found that the first four cells in a mouse embryo sometimes exhibit differences in histone methylation[9]. Petronis suggests that diverging epigenetic profiles might be what drives the cells to split into twins in the first place. His work on monozygotic twins has shown that the genomic loci that differ the most epigenetically are those involved in cell-division processes, which "may reflect an early developmental discordance as one of the hypothetical reasons of twin formation".

If that is the case, Reversade might not ever find a twinning gene. But that is not going to stop him from packing his bag for the twin town in Brazil. The armadillo gives him confidence that there are twinning genes to be found, as does a strain of zebrafish in which a mutation can generate a double head like that of conjoined twins. And so does the Jordanian family which, he says, "cannot be explained by these epigenetic phenomena but can be by conventional genetics".

What will he do if his results don't show a gene? "Publish them," he says simply. ■

David Cyranoski is *Nature*'s Asia-Pacific correspondent.

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