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RESEARCH REPORT

Diagnosis and high incidence of hyperornithinemia-hyperammonemia-homocitrullinemia (HHH) syndrome in northern Saskatchewan

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Abstract Mutations in the SLC25A15 gene, encoding the human inner mitochondrial membrane ornithine transporter, are thought to be responsible for hyperornithinemia-hyperammonemia-homocitrullinemia (HHH) syndrome, a rare autosomal recessive condition. HHH syndrome has been detected in several small, isolated communities in northern Saskatchewan (SK). To determine the incidence of HHH syndrome in these communities, a PCR method was set up to detect F188Δ, the common French-Canadian mutation. Neonatal blood spots collected from all newborns from the high risk area were genotyped for the F188Δ mutation for seven consecutive years. Using DNA analysis, we estimated that the heterozygote frequency for the mutant allele for HHH syndrome to be about 1 in 19 individuals, predicting one affected child with HHH syndrome for approximately every 1,500 individuals (1 in 1,550 live births; 1 child every 12 years) in this isolated population. The frequency for the mutant allele for HHH syndrome in this isolated community is probably the highest in the world for this rare disorder. We determined that ornithine levels, by tandem mass spectrometry, were not abnormal in newborns with F188Δ mutation, carriers and normals. Ornithine rises to abnormally high levels at some time after birth well past the time that the newborn screening blood spot is collected. The timing or the reasons for the delayed rise of ornithine in affected children with HHH syndrome have not been determined. Newborn screening for HHH Syndrome in this high risk population is only possible by detection of the mutant allele using DNA analysis.

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References to electronic databases: OMIM # 238970 (http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?cmd=entry&id=238970)

Competing interest None declared.

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Abbreviations

HHH	Hyperornithinemia-hyperammonemia-homocitrullinemia
ORNT1	Ornithine transmembrane transporter 1
bp	Base pair

Introduction

Hyperornithinemia-hyperammonemia-homocitrullinemia (HHH) syndrome is a rare, autosomal recessive inherited metabolic disorder (OMIM # 238970) of the urea cycle and ornithine degradation pathways. The condition arises due to mutations in the solute carrier gene SLC25A15 (MIM# 603861), encoding the inner mitochondrial membrane

ornithine transporter (ORNT1), which maps to 13q14 (Camacho et al. 1999).

More than 60 cases of HHH have been identified worldwide caused by 5 different mutations (Valle and Simell 2001; Fecarotta et al. 2006; Salvi et al. 2001; Mhanni et al. 2008; Miyamoto et al. 2001; Camacho et al. 1999, 2003, 2006; Tsujino et al. 2000; Korman et al.. 2004; Tessa et al. 2009). In Canada, HHH syndrome has been identified in the French-Canadian population (Camacho et al. 1999). The French-Canadian HHH mutation, designated as F188Δ (F188del; MIM # SLC25A15.0001), arises due to a deletion of codon 188 for phenylalanine resulting in an unstable, functionless protein. The 3-bp in-frame deletion occurs in a sequence of 4 consecutive TTC phenylalanine codons (bp 553–564) (Camacho et al. 1999) (GenBank: DQ896250.2, NCBI, NIH: www.ncbi.nlm.nih.gov/nuccore/123999346).

The pathophysiology of HHH involves diminished ornithine transport (in the periportal and pericentral hepatocytes and most peripheral tissues including skin fibroblasts) into the mitochondria with ornithine accumulation in the cytoplasm and reduced intramitochondrial ornithine causing impaired ureagenesis, hyperammonemia, and homocitrullinemia as well as orotic aciduria (Fig. 1). Homocitrulline is thought to originate from the transcarbamylation of lysine.

The clinical symptoms, resembling those of urea cycle disorders, range from protein-rich food intolerance, vomiting and lethargy to neurological signs such as spastic gait, pyramidal tract signs, myoclonic seizures, ataxia, coma and mental retardation (Valle and Simell 2001). Liver involvement is variable ranging from moderate fulminant hepatic failure (Fecarotta et al. 2006; Lemay et al. 1992; Mhanni et al. 2008; Tessa et al. 2009; Smith et al. 1992). Age of onset of symptoms is equally variable ranging from infancy to adulthood (Gray et al. 1995; Shih et al. 1992; Tuchman et al. 1990; Valle and Simell 2001; Tessa et al. 2009). Central nervous system impairment is thought likely to be secondary to the episodic hyperammonemia, although other uncommon biochemical abnormalities, such as polyamine metabolism, might be involved (Tessa et al. 2009). Chronic cytoplasmic accumulation of ornithine and other yet uncharacterized factors associated with ornithine metabolism (Valle and Simell 2001) are also observed. Treatment consists of restriction of protein intake, citrulline or L-arginine supplementation and use of sodium benzoate or sodium phenylbutyrate for hyperammonemia (Fecarotta et al. 2006; Mhanni et al. 2008; Valle and Simell 2001).

HHH syndrome can be detected by DNA analysis of the mutation concerned as well as through biochemical analysis by measurement of elevated ornithine, ammonia and homocitrulline levels. At the Saskatchewan Disease Control Laboratory, a polymerase chain reaction—capillary electrophersis (PCR-CE)-based assay, has been set up to detect the F188Δ mutation. Four of the five patients diagnosed in the laboratory have been identified using this technique. The three older patients were diagnosed prior to this assay setup. Four of them hail from one area of northern Saskatchewan, a region with a population of ~11,600. The population in this area is 94% Aboriginal[1] (Statistics Canada 2006a, 2006b)—predominately Metis, and Cree or Dene First Nations. The term Métis refers to descendants of mixed biological heritage, usually either French-First Nation or British/Scottish-First Nations (Brizinski 1993). Given the unique population demographics of northern Saskatchewan, the number of HHH children identified there, and the absence of data on the prevalence of HHH disease in this region, the incidence of HHH in these northern communities was measured by carrying out retrospective and prospective screening of newborn blood spots for the F188Δ mutation. This survey also allowed us to compare the performance of our novel PCR-CE method and the ability of the existing newborn screening program in Saskatchewan to detect HHH Syndrome by tandem MS using elevated ornithine levels.

Methods

Population and geographic location selection

Three small towns (Towns A, B, and C) were chosen to represent the affected region of northern Saskatchewan. All newborn blood spot cards, submitted to the provincial newborn screening program, with maternal residency address listed as one of the three towns, were enrolled in the study. Except for the physical resident location, no other identifiers were used. Blood spots dating as far back as 1999 were retrieved for the retrospective samples. Newer blood spots from the same three towns were obtained following informed consent. The study period lasted from August 1999 to January 2007. Three (3 mm or 1/8 inch diameter) circles were punched-out from dried blood spot into a 1.5-mL micro-centrifuge tube and stored at 4°C for analysis. Ethics approval was obtained from the University of Saskatchewan ethics review committee and the appropriate health authorities.

[1] Aboriginal Peoples is a collective name for all of the original peoples of Canada and their descendants. The *Constitution Act* of 1982 specifies that the Aboriginal Peoples in Canada consist of three groups— Indians, Inuit and Métis. In Canada, many people prefer to be called First Nations or First Nations People instead of Indians (National Aboriginal Health Organization's Terminology Guide. www.naho.ca/english/pdf/terminology_guidelines.pdf)

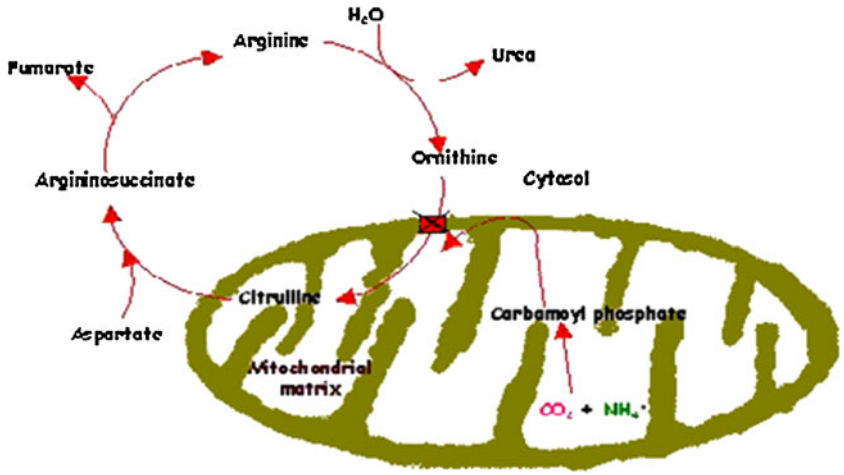

Fig. 1 Metabolic pathways of the urea cycle. The ORNT1 membrane transporter is indicated by the *crossed red box*

DNA extraction and analysis

Genomic DNA was extracted from the blood spots using the dried blood spot protocol of a commercial DNA extraction kit (QIAamp DNA mini kit; Qiagen, Mississauga, ON, Canada). Upon DNA extraction, the ORNT1 reading frame (consisting of 362 base pairs spanning base pair 553–564 of 13q14) was amplified using DV3653 (forward 5′-CTGCTTCAGGCCCTGTCC TAA.-3′) primer and DV 3620-FAM (reverse 5′-CTTAG GAAAGATGGCCC-3′) primer. For PCR amplification, a sample reaction containing 25 μl total volume (consisting of 1X PCR buffer II, 2.50 mM $MgCl_2$, 0.20 mM each dNTPs, 0.50 μM DV3620-FAM primer, 0.50 μM DV3653 primer, 1 unit AmpliTaq Gold polymerase, 1 μl DNA sample) was incubated in a thermal cycler (ABI 9700; Applied Biosystems, Mississauga, ON, Canada) under the following conditions: 94°C for 10 min (once), [95°C for 10 s, 55°C for 10 s, 72°C for 30 s] (40 cycles), 72°C for 10 min, and finally 4°C indefinitely.

Briefly, following denaturation, fluorescently labeled PCR products were resolved by capillary electrophoresis through POP-7 polymer in a 36 cm capillary array using an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems). Fragment sizes were determined by analysis with GeneMapper software (ver 4.0) using GS-500 Rox as an internal standard (Applied Biosystems). Fragment sizes resolve as either 359 or 362 bp depending upon which allele is amplified.

Tandem mass spectrometric analysis

Amino acids, acylcarnitines and internal standards were analysed on an API-2000 tandem MS connected to an Agilent 1100 HPLC with a Wellplate autosampler as described previously (Chace et al. 1997). Samples were injected directly, without chromatography, at 100 μL/ min. A total of 25 μL was injected for analysis from each sample or standard. The API-2000 was operated using the TurboIon Source in electrospray ionisation mode. The various acylcarnitines and amino acids were quantitated in MRM mode by relating the ion abundance of each compound to a stable isotope-labeled internal standard. The various transitions and the calculation of the quantity of each analyte were determined using the Analyst v. 1.2, and the Chemoview software supplied by the manufacturer (Applied Biosystems).

Data analysis

Results obtained were recorded on a Microsoft Excel spreadsheet. The HHH genotype frequency was calculated using the Hardy-Weinberg equilibrium model: (Bodmer 2001) $p^2 + 2pq + q^2 = 1$, where, p^2 is the predicted frequency of wild-type individuals in the population, $2pq$ is the predicted frequency of carrier individuals, q^2 is the predicted frequency of mutant individuals, and Pearson's χ^2 test for the deviation of the population from the model (null hypothesis that the population is in Hardy–Weinberg equilibrium). The allele frequency was a calculation from the analysis of the data assuming complete ascertainment. The predicted homozygote genotype frequency would be calculated based on the likelihood of 2 carriers mating, i.e. $2pq \times 2pq \times 1/4$ or using the Hardy–Weinberg equation $p^2 + 2pq + q^2 = 1$.

Results

The birth statistics for the study period are listed in Table 1. The study catchment area had 126 recorded live births for this period. The combined population of the three towns was 3,056 (Saskatchewan Ministry of Health 2008).

Table 1 2008 birth statistics for the study area in northern Saskatchewan

Town	Live births	Population
A	30	1,412
B	28	1,522
C	68	3,056
Total	126	5,990

F188Δ genotype analysis resulted in three genotypes: normal individuals (wild-type) with two alleles of 362 bp each, carriers with 362 bp and 359 bp alleles, and affected individuals (mutants) with two alleles of 359 bp each. A total of 657 newborn blood spots were analyzed (from August 1999 to January 2007). Of these, 628 were identified as wild-type for the F188Δ mutation, 25 as carriers and 4 as mutants (Table 2). Applying the Hardy–Weinberg equation (Appendix A; Eq. 1) to the data, the frequency of carriers ($2pq$) in the population was estimated at 1 in 19 ($2pq$=0.0504) and that of HHH (q^2) at 1 in 1,492 individuals (q^2=0.0007).

The frequency of the mutant allele (q) in the population was estimated at 1 in 38 individuals (q=0.0259). Therefore, the likelihood of a mutant child being born in the community was predicted as approximately 1 in 1,550 births ($2pq \times 2pq \times 1/4$). Based on Table 1 (birth rate of 126 live births/year), one (1) child with HHH syndrome would be born every 12 years.

A Pearson's χ^2 test for deviation of the Hardy–Weinberg equilibrium (Appendix B) determined that the population was not in equilibrium (p>0.05). This is suggestive of a loss of genetic variation due to either a small population size, or assortative pairing, or both.

All the HHH-affected individuals were genotyped on clinical presentation of the disease. This was due to the fact that all of them were born prior to the start of the project. One child born outside of the study area was also identified as mutant with F188Δ mutation by genotyping in our laboratory on presentation (Mhanni et al. 2008). Both this child and our latest identified infant with HHH had normal ornithine and arginine levels during the newborn screening period, and thus were never flagged for further investigations. When they presented with clinical symptoms, the ornithine levels were 933 and 580 μmol/L (reference range= 40–160 μmol/L), respectively. Figure 2 shows orthinine levels during the newborn period and Fig. 3 shows ornithine

Table 2 Results of F188Δ genotype analysis

Genotype	Wild-type	Carrier	Mutant	Total
Number	628	25	4	657

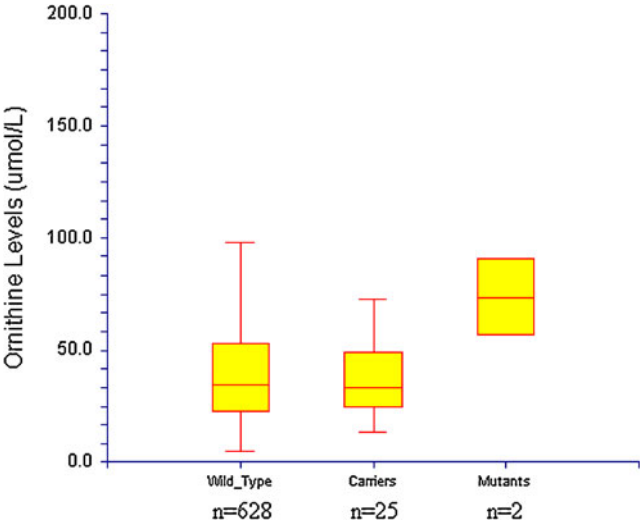

Fig. 2 Box-and-whisker plots demonstrating newborn ornithine levels in the three genotypic groups. The mutant results are based on a group size of two (both born outside study area; one of them from a different province but diagnosed in our laboratory and the other within the province). Newborn blood spots of the older four individuals were unsuitable for ornithine level determination by tandem mass spectrometry due to the age of the blood spots. These individuals were born prior to the implementation of tandem mass spectrometry. All other groups had ornithine levels within normal value (<120 μmol/L). The *horizontal line* in each *box* represents the median, with the *boxes* representing the interquartile range

levels in the five patients identified in our laboratory with HHH Syndrome at various ages following birth. All the levels are elevated except the one collected in the newborn period. Newborn ornithine levels for the four older mutants were not available due to the unsuitability of the blood spots for this analysis. The blood spots were older than 8 years.

Ornithine levels during the newborn period were not different between the wild-type and carrier groups (p= 0.7673, Fig. 2). Comparative statistics were not calculated between the other groups and the mutant ornintine levels due to the small size of the group. However, none of the genotypes (wild, carrier and mutants) had abnormal ornithine levels during the newborn period (relative to the ornithine cutoff of 120 μmol/L).

Discussion and conclusions

HHH syndrome is a rare inherited metabolic disease. In Canada, it is found with a higher incidence among the French-Canadian population. The F188Δ genotype is responsible for this disease in this population (Camacho et al. 1999). In the three communities in northern Saskatchewan where this study was conducted, Métis are predominantly of French-Canadian-Cree or French-Canadian-Dene origin and make up about 66% of the population with 26% being First

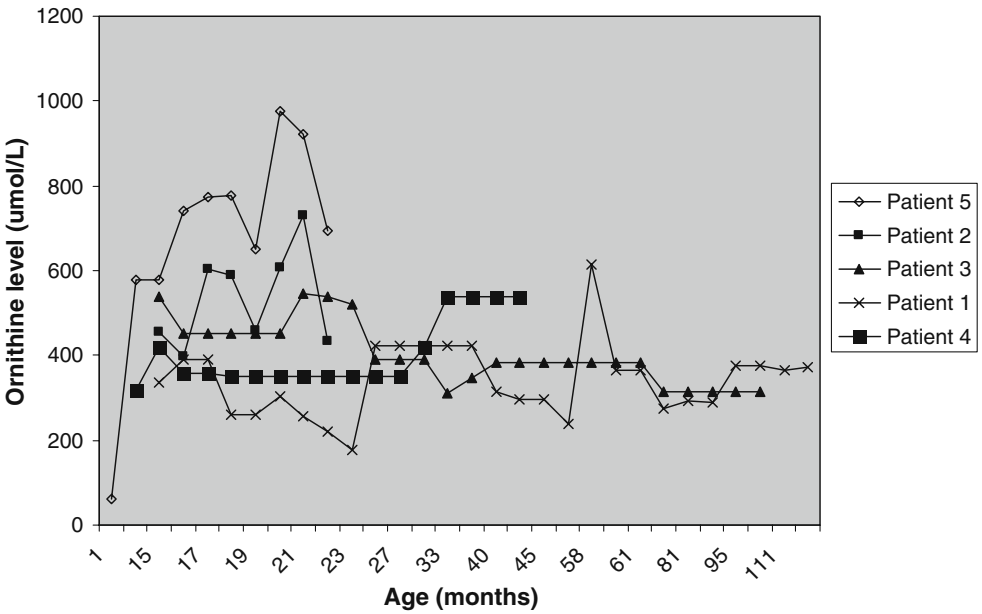

Fig. 3 Ornithine levels in five patients with HHH syndrome. *Patient 1* is the oldest and first diagnosed with HHH. *Patient 5* is the youngest and most recent diagnosis. All except patient 5 have ornithine levels starting at presentation of HHH. Patient 5, born outside the study area, was diagnosed after start of the newborn screening using tandem mass spectrometry. Thus, ornithine levels were available. The other patients were diagnosed prior to the program. Newborn ornithine levels were unavailable

Nations (2006 Aboriginal Peoples Survey). The presence of this gentoype in this part of northern Saskatchewan may be from an extension of the French Canadian founder effect reported for HHH Syndrome (Debray et al. 2008). The region of northern Saskatchewan consists of small isolated communities that adhere to a relatively traditional way of life. These communities consist of distinct groups of people (Cree, Dene and Metis). Population migration is to a minimum, often facilitating consanguinity.

We had earlier suspected that HHH syndrome is over represented in this region. Our study shows that the frequency of HHH (1 in 1,492 individuals); of carriers (1 in 19) and, the associated predicted outcome of producing a child affected with HHH syndrome (~1 in 1,550 live births equating to 1 child every 12 years) in this region is high for a rare genetic disease. This finding is underscored by the fact that the population is not in Hardy–Weinberg equilibrium (χ^2=30.83, p<0.05), possibly because of the small population size. For these northern communities, the physical isolation, way of life and cultural heritage would favor the factors tending to population disequilibrium of the Hardy–Weinberg calculation.

This is the first study to be conducted that quantifies the incidence of HHH syndrome this area of northern Saskatchewan. Although global surveys of HHH Syndrome have been conducted (Camacho et al. 1999), no other study has looked at the penetrance of F188Δ mutation (or any other mutation) in any community. Initial clinical symptoms (failure to thrive, developmental delay, and liver dysfunction) in patients with HHH syndrome are often nonspecific, facilitating misdiagnosis if plasma amino acid levels are not performed (Debray et al. 2008). Given these findings, it is imperative to have a population screening strategy (of the F188Δ mutation) in these communities. Genetic screening using the same dried blood spot as the newborn screening program would not only lower any additional logistics and costs to the health care system but would also provide a guaranteed delivery of diagnostic services to these communities. Newborn screening by tandem mass spectrometry, which has been in operation in Saskatchewan since 2001, was looked at as an obvious way of detecting HHH syndrome prior to the detection of symptoms. As our data show, ornithine levels at birth are within normal limits in carriers and mutants (although newborn ornithine levels were only obtained for two individuals, one from the same province but outside the study area and the other from out of province). However, this finding of normal ornithine levels in mutants (affected infants) during the newborn period is not shared by other workers (Shih et al. 1992; Zammarchi et al. 1997). In this light, it is important to interpret our findings in this group with caution given the small size of the study group (n=2). Nevertheless, biochemical screening of HHH syndrome using newborn ornithine levels appears to be unreliable. Genetic screening during this period maybe the only effective tool. Reasons for the low ornithine levels at birth in babies affected with HHH syndrome are unclear. A review of the literature indicates that a somewhat similar situation has been documented in a patient with gyrate atrophy and also in a mouse model of ornithine-delta-aminotransferase deficiency (Wang et al. 1996). Wang and co-workers have shown that, both in their mouse model and in a human infant diagnosed in a prenatal fashion, ornithine and arginine levels were low for the first 100 days of life. Once the diet was supplemented with modest amounts of arginine, the child developed hyperornithinemia, and the hypoargininemia was also corrected (Wang et al. 1996). This data suggests that the marginal ammonium detoxification capability during the early days of life is sufficient to

maintain low levels of ornithine and arginine. In human infants with HHH syndrome, a similar situation may be the case. Further studies will be required to elucidate the biochemical and dietary changes that occur in human infants with HHH syndrome.

Appendix A: Hardy–Weinberg Equation

Hardy-Weinberg equation

$$P^2 + 2pq + q^2 = 1 \quad (1)$$

Using the total population number of 5,990 and a confirmed diagnosis of HHH in 4 children from the area, Frequency of HHH in the three communities, q^2, is:

$$q^2 = 4/5990 = 0.00067 (1/0.00067 = 1492.54;\ \text{i.e.,}\ \sim 1 \text{ in } 1500 \text{ individuals})$$

Frequency of the mutant allele, q, is:

$$q = \sqrt{0.00067} = 0.0259 (1/0.0259 = 38.61;\ \text{i.e., } 1 \text{ in } 38 \text{ individuals})$$

Frequency of normal allele, p, is:

$$p = 1 - q = 1 - 0.0259 = 0.9741 (97\%)$$

Frequency of heterozygote, $2pq$, is:

$$2pq = 2(0.9741 \times 0.0259) = 0.0504 (1/0.0504 = 19.84;\ \text{i.e., } 1 \text{ in } 19.8 \text{ individuals})$$

Based on 657 births, we should have found 33 heterozygotes (657/19.8)

The likelihood of two (2) carriers mating to produce an affected off-spring is:

$$2pq \times 2pq \times 0.25 = 0.000635 (1/0.00064 = 1562.5;\ \text{i.e.,}\ \sim 1 \text{ in } 1550 \text{ births})$$

Based on Table 1 (birth rate of 126 live births/year), one (1) child with HHH syndrome will be born every 12 years (1550 births/126 births/year).

Appendix B: test for deviation from Hardy–Weinberg equilibrium

The Hardy-Weinberg expectation (Exp) of each genotype is given by the sum of the probability of each genotype multiplied by the total. Thus, in our study, using the results from Table 2 (Results of F188Δ genotype analysis), we obtain the following:

$$\begin{aligned}
\text{Exp (wild genotype)} &= p^2 n = (0.9741)^2 \times 657 \\
&= 0.9489 \times 657 = 623.43 \\
\text{Exp (carrier genotype)} &= 2pqn = 2 \times (0.9741 \times 0.0259 \times 657) \\
&= 2 \times 16.58 = 33.16 \\
\text{Exp (mutant genotype)} &= q^2 n = (0.0259)^2 \times 657 = 0.44
\end{aligned}$$

Pearson's chi-square (X^2) test, at 1 degree of freedom (number of genotype−number of alleles=3−2=1) and 5% significance level is given by the following:

$$\begin{aligned}
X^2 = {} & [\text{number of wild genotype} - \text{Exp(wild genotype)}]^2 \Big/ \text{Exp(wild genotype)} \\
& + \\
& [\text{number of carriers} - \text{Exp(carrier genotype)}]^2 \Big/ \text{Exp(carrier genotype)} \\
& + \\
& [\text{number of mutants} - \text{Exp(mutant genotype)}]^2 \Big/ \text{Exp(mutant genotype)}
\end{aligned}$$

Thus, for our study group,

$$\begin{aligned}
X^2 &= [628 - 623.43]^2 \Big/ 623.43 + [25 - 33.16]^2 \Big/ 33.16 \\
&\quad + [4 - 0.44]^2 \Big/ 0.44 \\
&= (20.88/623.43) + (66.56/33.16) + (12.67/0.44) \\
&= 0.033 + 2.01 + 28.79 \\
&= 30.83
\end{aligned}$$

The 5% significant level for 1 degree of freedom is 3.84 (from X^2 table). Since the calculated X^2 value (30.83) is greater than this value (30.83 vs 3.84), the null hypothesis (the population is in Hardy-Weinberg equilibrium) is rejected.

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