Distinct gut virome profile of pregnant women with type 1 diabetes in the ENDIA study

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ABSTRACT

Background. The importance of gut bacteria in human physiology, immune regulation and disease pathogenesis is well established. In contrast, the composition and dynamics of the gut virome are largely unknown; particularly lacking are studies in pregnancy. We used comprehensive virome capture sequencing to characterize the gut virome of pregnant women with and without type 1 diabetes (T1D), longitudinally followed in the Environmental Determinants of Islet Autoimmunity study.

Methods. In total, 61 pregnant women (35 with T1D and 26 without) from Australia were examined. Nucleic acid was extracted from serial fecal specimens obtained at prenatal visits and viral genomes were sequenced by virome capture enrichment. The frequency, richness and abundance of viruses were compared between women with and without T1D.

Results. Two viruses were more prevalent in pregnant women with T1D: picobirnaviruses (odds ratio 4.2, 95% confidence interval 1.0–17.1; p=0.046) and tobamoviruses (3.2, 1.1–9.3; p=0.037). The abundance of 77 viruses significantly differed between the two maternal groups (≥2-fold difference; p<0.02), including eight *Enterovirus B* types present at a higher abundance in women with T1D.

Conclusions. These findings provide novel insight into the composition of the gut virome during pregnancy and demonstrate a distinct profile of viruses in women with T1D.

KEYWORDS

enteric virus; feces; gut virome; pregnancy; type 1 diabetes; virome capture sequencing
BACKGROUND

Pregnancy is a complex immunological state in which the balance between an inimical alloimmune response and an environment of maternal tolerance may be perturbed by virus infections\(^1\), potentially resulting in significant perinatal morbidity\(^2\). Helper T-lymphocyte (Th) associated cytokines shift from Th1 towards Th2 to protect the fetus, however, this Th2 bias can diminish cell-mediated immunity and increase vulnerability to intracellular infections, including viruses\(^3-5\). In pregnant women with type 1 diabetes (T1D), hyperglycemia may impede pathogen clearance, increasing the duration of gestational infections\(^6\) and heightening the risk of adverse outcomes in the fetus, including subsequent development of T1D during childhood\(^7\). This is supported by our recent systematic review and meta-analysis of 2,992 women and children, which demonstrated a significant association between maternal virus infection in pregnancy and T1D in offspring (odds ratio (OR) 2.2, 95% CI 1.2-3.8; \(p=0.008\))\(^8\). To date, no study has examined the gut virome during pregnancy, and how it may be altered by T1D.

METHODS

Study population and sample selection. The gut virome of 61 pregnant women (35 with T1D and 26 without) in the Environmental Determinants of Islet Autoimmunity (ENDIA) study was examined (Supplementary Table 1). ENDIA is a prospective cohort study of children at risk of T1D (have \(\geq 1\) first-degree relative with T1D), followed longitudinally from pregnancy to 3 years of life\(^9\). Women recruited between 2012 and 2016 were included in this analysis. Overall, 59 pregnancies were singleton and two were twin pregnancies. Fecal samples were collected during the first (\(n=18\)), second (\(n=47\)) and/or third trimester of pregnancy (\(n=59\), which were defined as gestational age of 1-14, 15-26 and 27-42 weeks, respectively. All samples were stored at -80°C in aliquots prior to
analysis. For every participant, all available samples were examined. In total, 49/61 women had samples for multiple timepoints and 12/61 had samples for all three trimesters.

The study was reviewed and approved (13th July 2016) by the study’s lead Human Research Ethics Committee at the Women’s and Children’s Health Network under the National Mutual Acceptance Scheme (HREC/16/WCHN/66) and at all participating study sites in Australia. All participants provided written informed consent and were free to withdraw from the study at any time. Families were excluded if the mother could not comprehend her participation in the study and therefore was unable to provide informed consent.

**Nucleic acid extraction.** Total nucleic acid (NA) was extracted using the MagMAX Total NA Isolation Kit (Thermo Fisher Scientific) on the semi-automated KingFisher FLEX Purification System (Thermo Fisher Scientific), following manufacturer’s guidelines with minor modifications. 30% (w/v) fecal suspensions were prepared in 1 x PBS and centrifuged 5 min. All spin steps performed at 13,000 x g at room temperature. Following centrifugation, 175 μL of supernatant was transferred to zirconium bead tubes containing 235 μL Lysis/Binding Solution. Bead tubes were shaken at 2,400 rpm on the Bioshake iQ (Quantifoil Instruments, Jena, Germany) for 15 min, then centrifuged 3 min. Into new tubes, 300 μL lysate was transferred and further centrifuged for 6 min. Total NA purified from 200 μL of lysate and stored at -80°C.

**Sequence-independent amplification.** Total NA was subjected to cDNA synthesis and sequence independent pre-amplification (SIP) using the Transplex Complete Whole Transcriptome Amplification Kit (Sigma-Aldrich, WTA1, St. Louis, MO), following a published protocol10. Briefly, 3.5 μL of total NA was denatured at 95°C for 5 min instead of 70°C before cDNA synthesis to ensure amplification of both DNA and RNA molecules. Denatured NA was cooled to 18°C and cDNA was synthesized using the following thermocycling conditions: 18°C 10 min, 25°C 10 min, 37°C 30 min, 42°C 10min, 70°C 20 min and 4°C holding. The entire cDNA library was used as template for SIP using
following cycling conditions repeated 22 times: 94°C 30 s and 70°C 5 min. Following amplification, PCR products were visualized on an agarose gel prior to purification using the ChargeSwitch-Pro PCR CleanUp Kit (Thermo Fisher Scientific).

**Virome capture sequencing.** 1 µg of dsDNA was used for library synthesis using the KAPA Hyperplus kit (KAPA Biosystems, Wilmington, MA) with single-index adapters. Briefly, dsDNA was enzymatically fragmented to an average of 200 bp. Fragments were purified using AmpureXP beads (Beckman Coulter, Brea, CA). Libraries were amplified 6-9 cycles, quality checked on the LabChip GX Touch 24 Bioanalyzer (PerkinElmer, Waltham, MA) and quantified using the picogreen assay (Thermo Fisher Scientific) on the Victor X2 Fluorescent Microplate Reader (PerkinElmer). Completed libraries were pooled by equal mass for sequence capture. VirCapSeq-VERT was performed according to the Nimblegen SeqCap protocol (Roche, Basel, Switzerland) as described previously. Post-capture libraries were purified and amplified before sequencing. To ensure sufficient depth of coverage (approximately 10 million unique sequence reads/sample), uniquely barcoded samples were pooled at a maximum of 20 libraries per pool (20-plex) and sequenced on a lane of HiSeq 4000 (Illumina, San Diego, CA).

**Genome sequence analysis.** Genome assembly, contig generation and taxonomic classification of reads were performed as previously described. De-multiplexed and quality-trimmed sequence reads were aligned against host reference databases from GenBank (NCBI) using the Bowtie2 mapping algorithm (version 2.1.0) to remove the host background. Filtered reads were assembled de novo using either SOAPdenovo2, MEGAHIT or MIRA assemblers, then contigs and unique singletons were subjected to homology search at the nucleotide level using MegaBLAST. Sequences that exhibited poor or no homology at the nucleotide level were screened further by BLASTX against the viral GenBank protein database. Viral sequences detected from BLASTX analysis were subjected to another round of BLASTX homology search against the entire GenBank protein database to correct for biased E values and inaccurate taxonomic classifications. For reference-based alignments,
to visualize depth and spread of coverage for individual viruses, both Integrated Genomics Viewer\textsuperscript{16} and Geneious (version 9.0.5)\textsuperscript{17} was used. Following taxonomic classification, read counts were corrected to account for sample bleeding due to Illumina index cross-talk, where sequences with single index barcodes are erroneously sorted, resulting in approximately 0.1% of total reads being distributed to the incorrect sample Fastq file. Cut-offs were applied across each pool separately. For each virus, 0.1% of the highest read count in that pool was calculated and subtracted from the number of reads of that virus in each sample. All resulting read counts below one were corrected to zero. This process minimized the risk of false ‘positive’ identification of viruses in samples. To evaluate virus positivity, a threshold of 100 viral reads matched by BLAST at the species level, randomly distributed over the target genome, was applied. This threshold was selected for its proximity to the typical limit of detection of targeted qPCR (~100 viral copies/mL), determined based on previous VirCapSeq-VERT experiments using whole blood\textsuperscript{11} and feces (unpublished data).

**Statistical analysis.** The STROBE reporting guidelines for observational cohort studies were followed\textsuperscript{18}. Continuous demographic variables are reported as a mean ± standard deviation (SD) for parametric data and median [IQR] for skewed data, and categorical data as number (%). Participant characteristics, including demographic variables, lifestyle factors and comorbidities, are reported according to T1D status and were compared using independent t-tests and Fisher’s Exact tests for continuous and categorical data, respectively. The socioeconomic index for areas (SEIFA) (index for relative socioeconomic disadvantage) percentile for the postal area in which each patient resided was used as an indicator of socioeconomic status\textsuperscript{19}. High SES was defined as >75\textsuperscript{th} percentile\textsuperscript{20}. Virus positivity was determined by a positivity threshold of 100 viral reads matched by BLAST at the species level.

The differential abundance of viruses between mothers with and without T1D was examined using the edgeR package (version 3.14.0)\textsuperscript{21} in R (version 3.3.0). A matrix of read counts, corrected for index cross-talk, was generated encompassing all samples and detected viruses. Each matrix entry had a
count of one added to avoid issues with division by, or log function of zero\textsuperscript{22} before conversion to counts per million (CPM). Data were normalized using the Relative Log Expression method with respect to library size\textsuperscript{23}. Two methods, common and tag-wise\textsuperscript{24}, were used to estimate the biological coefficient of variation. Samples were divided into case and control groups, and the “exact” test used to perform hypothesis testing\textsuperscript{25}. P-values were adjusted to control false discovery rates with the Benjamini-Hochberg multiple testing correction procedure\textsuperscript{26}. Viruses with an adjusted p<0.05 were identified as displaying statistically significant differential abundance between case and control groups. Heatmaps were created to visualize the data using iheatmapr package in R\textsuperscript{27}.

The richness of vertebrate-infecting viruses in feces was estimated using EstimateS software (version 9.1.0)\textsuperscript{28}. Estimates used to calculate richness at the genus level and sample-based rarefaction curves were computed using pre-published analytical formulas\textsuperscript{29}. In total, 100 randomizations (runs) were completed, extrapolating by a factor of 1.0 with estimates (knots) at every sample. Estimates of vertebrate-infecting virus richness in cases were compared with those of controls using a non-parametric test, assuming the estimates in cases and controls are statistically independent of each other. Rarefaction curves were plotted in Microsoft Excel (version 15.3, Redmond, WA).

The association between maternal T1D and positivity for each virus detected in mothers during pregnancy was examined using generalized estimating equations (GEE). The GEE method for the binary outcome of virus positivity at the genus level (logit link function) was applied to account for the correlation among longitudinal observations of the same participant. In the GEE model, virus infection at the genus level was the independent variable and the major dependent variable was maternal T1D. Other explanatory variables examined were maternal age at conception, parity, pet ownership, SES, maternal body mass index (BMI), education, maternal smoking, and age at sample in the model.
Univariate and multivariable analyses were undertaken on both study groups. These covariates and factors in the GEE model were selected from a larger set of potential covariates by forwards and backwards regression. An individual model was used for each genus of virus, with independent variables that exhibited association with the outcome variable being included in multivariable analysis. GEE models were compared using the quasi-likelihood under independence model criterion (QIC), and the lowest scoring and most parsimonious models are reported. An exchange correlation structure was utilized and results expressed as odds ratio (OR) determined by the regression coefficient $\exp\beta$, with 95% confidence intervals (CI). Frequency of virus positivity limited multivariable analysis to the five most frequent genera of virus detected in both study groups. Statistical analyses were performed using IBM SPSS Statistics (version 24.0, Chicago, IL). Statistical significance was defined as $p \leq 0.05$.

**RESULTS**

The pregnancy gut virome of women with and without T1D was characterized using VirCapSeq-VERT on 124 fecal specimens collected from 61 mothers ($n=35$ with T1D, $n=26$ without T1D) in the ENDIA study (Supplementary Table 1). The mean (± SD) age of mothers at conception was $32 \pm 4$ years and BMI was $27 \pm 6$ kg/m$^2$. Compared to women without T1D, women with T1D gave birth at a significantly younger age, after a shorter gestational length and had fewer children (Supplementary Table 1).

High-throughput sequencing generated ~2 billion raw reads, which reduced to 1.6 billion reads following filtration of host and primer sequences. This equated to $12.7 \pm 4.2$ million filtered reads per sample. In total, 29 genera of eukaryotic viruses were detected and 63% of samples (78/124) tested positive for at least one virus (Figure 1). Members of the *Picobirnavirus*, *Parechovirus* and *Enterovirus* (EV) genera were among the most frequent vertebrate-infecting viruses sequenced.
Although non-vertebrate-infecting viruses were excluded from VirCapSeq-VERT enrichment, tobamoviruses were frequently detected, suggesting that plant viruses are highly abundant in the gut during pregnancy, and prevalent in human feces. Whilst not reaching statistical significance, there was a trend to higher virus positivity in mothers with T1D versus without (64% vs 50%; p=0.14). Rarefaction analysis revealed no difference in the richness of vertebrate-infecting viruses between women with and without T1D (Supplementary Figure 1), suggesting that all participants were exposed to a comparable community of viruses, independent of their T1D status.

Examination of longitudinal samples from 49/61 participants (n=28 with T1D, n=21 without T1D) identified alphapapillomaviruses, circoviruses, parechoviruses, and picobirnaviruses in multiple trimesters of pregnancy within individuals (Figure 2). This may indicate persistent or recurring infection by closely related strains. There was no difference in the proportion of virus positive samples across the three trimesters (p=0.95). Chicken anaemia virus, genus Gyrovirus, and pepper mild mottle virus, genus Tobamovirus were also detected across multiple trimesters but these most likely originated from dietary intake. Two viruses were more prevalent in women with T1D: picobirnaviruses (33% vs 9%; OR 4.2, 1.0-17.1; p=0.046) and tobamoviruses (22% vs 9%; OR 3.2, 1.1-9.3; p=0.037). In multivariable generalized estimating equation (GEE) models, the higher odds of having picobirnaviruses and tobamoviruses in women with T1D remained significant after adjustment for maternal age. Also, there was a trend towards higher rates of gyroviruses, chloroviruses and carlaviruses in women with T1D, but differences did not reach statistical significance. The frequency of EV did not differ between the two maternal groups, however significant differences in EV types were observed. Coxsackievirus A2 (CVA2), CVB4, CVB5, Rhinovirus B and ECHOviruses were detected exclusively in women with T1D, whilst CVA6, CVA10, CVA14 and EV71 were present only in mothers without T1D (Supplementary Table 2).

Differential abundance analysis identified 77 virus types with ≥2-fold significant difference (p<0.02) between pregnant women with T1D versus those without, with a false discovery rate <5% (Figure 3...
Among the top 15 differentially abundant viruses were three EV-B types (CVB4, CVB3 and ECHOvirus E18), all present at higher abundance in women with T1D (Table 1). Conversely, four EV-A types (CVA10, CVA16, CVA5 and CVA14) were more abundant during pregnancy in women without T1D.

DISCUSSION (985 words)
We demonstrated that eukaryotic viruses are prevalent in the gut of women during pregnancy, and that women with T1D are more likely to harbor picobirnaviruses and tobamoviruses compared to women without T1D. Furthermore, we found significant differences in viral abundance between women with and without T1D, including eight Enterovirus B types that were all present at a higher abundance in women with T1D. These results demonstrate a distinct profile of viruses in women with T1D in pregnancy.

The pathogenicity of picobirnaviruses in humans remains to be definitively established. A weak association with gastroenteritis in animals has been found, whereas in humans they are only considered as possible opportunistic pathogens. Most recently, picobirnaviruses were detected at high levels in patients with HIV and graft-versus-host disease, leading to the proposal that they may serve as a biomarker of immunosuppression. Thus, it is plausible that a higher prevalence of picobirnaviruses in women with T1D could be reflective of impaired antiviral defence.

Tobamoviruses are not known to be pathogenic to humans and are commonly thought to be introduced to the gut through diet. Therefore, their higher prevalence in women with T1D during pregnancy may reflect differences in diet or consumption of contaminated drinking water.

Alternatively, there may be other factors involved such as gut permeability and intestinal inflammation, which are both increased in individuals with T1D, may prevent effective clearance of
dietary viruses. Our frequent detection of tobamoviruses in feces is consistent with other virome studies, including a recent study of five mother-infant pairs.\textsuperscript{30,37}

The predominance of EV-B types found in pregnant women with T1D is consistent with higher rates of EV-B observed in individuals with T1D versus healthy controls.\textsuperscript{38-40} Furthermore, the greater abundance of CVB4 in women with T1D complements the body of molecular and epidemiological evidence implicating CVB4 in T1D pathogenesis.\textsuperscript{39,41-44} Conversely, EV-A types were more prevalent and present at a higher abundance in pregnant women without T1D (Figure 3). This contrasts results obtained from children in the Finnish Diabetes Prediction and Prevention (DIPP) study that found a higher rate of EV-A infections in cases who developed T1D compared with matched controls, during a time window more than 12 months prior to the first detection of islet autoantibody.\textsuperscript{45} The result also contrasts our own findings in the Australian Viruses In the Genetically at Risk (VIGR) study,\textsuperscript{46} where we also found a predominance of EV-A viruses in feces of children prior to or at the time of the first islet autoantibody detection when compared to matched controls (Kim et al, Scientific Reports, in press).\textsuperscript{47}

A recent study examining the intestinal virome changes that precede the development of autoimmunity in T1D-susceptible children detected circoviruses at a greater abundance and prevalence in controls, suggesting that infection with this virus may offer protection from the development of T1D.\textsuperscript{48} Consistent with this hypothesis, circoviruses were exclusively detected in women without T1D in our investigation (Figure 1). However, our sample size was too small to detect a statistically significant difference in frequency and the case participants examined in our study were all women who had a long-standing T1D.

We examined potential confounding factors, in addition to T1D, that may influence the risk of virus infection during pregnancy. In univariate analysis, younger maternal age was associated with picobirnaviruses, older maternal age and no tertiary education with EVs, and low socioeconomic
status with gyroviruses. In multivariable GEE models, all aforementioned relationships remained significant except between maternal age and picobirnaviruses. Glycemic control may also influence susceptibility to infection, however the majority of women with T1D in our study achieved glycemic targets for pregnancy.

To the best of our knowledge, this is the first study to examine the longitudinal gut virome across all three trimesters of pregnancy, providing novel baseline data for future gut virome investigations. Another major strength of this study is the application of virome capture sequencing, which is the most sensitive and comprehensive sequence-based virome characterization tool currently available for vertebrate-infecting viruses. This method specifically targets all known viruses capable of infecting humans and other vertebrates, significantly reducing sequences produced from host and bacterial background, allowing up to a 10,000-fold increase in the number of viral reads recovered compared to conventional virome sequencing methods. Furthermore, our method enabled the examination of both RNA and DNA viruses simultaneously. Despite these strengths, the interpretation of our virome data is limited by the fact that sequencing cannot differentiate between the presence of viral genomes in the gut versus actively replicating viruses. In addition, the absence of a non-pregnant control group precluded the analysis of the effect of pregnancy on virus infection in this study.

Given the results of our recent systematic review and meta-analysis of 2,992 women and children that demonstrated a significant association between maternal virus infection in pregnancy and T1D in the offspring, future studies could be aimed at examining the impact of maternal virus infections on the development of islet autoimmunity and T1D in the offspring. For this purpose, the offspring of women examined in this study are being followed longitudinal for these two outcomes as part of the ENDIA study, a prospective cohort study following at risk children. The characterization of the gut virome in these mother-infant pairs will allow identification of potential vertical transmission of viruses (currently underway). The impact of diet on the gut virome will also be examined. The
virome of other potential sources of vertical transmission should also be investigated such as the oral, skin, breastmilk and the vaginal virome, which has been recently shown to be of clinical importance for its potential contribution to preterm birth\textsuperscript{50}.

In conclusion, our findings provide novel insight into the diversity and dynamics of the gut virome during pregnancy and identify T1D and maternal age as key factors influencing virus infection in pregnancy. We show a novel potential association between T1D and picobirnaviruses and demonstrate a distinct profile of viruses during pregnancy in women with T1D, providing novel targets for prevention studies.

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\section*{Acknowledgments}

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Conflict of Interests

The authors report no conflict of interest.
REFERENCES


**Table 1.** Top 15 differentially abundant species of viruses between the gut of pregnant women with type 1 diabetes versus without.

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Paramecium bursaria Chlorella virus  
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Coxsackievirus A5  
-6.4  4.30E-28  4.80E-27  13
Coxsackievirus A14  
-6.4  4.60E-28  4.80E-27  14

Abbreviations: FD, fold difference; FDR, false discovery rate; T1D, type 1 diabetes.

FIGURE LEGENDS

Figure 1. Viruses detected using VirCapSeq-VERT. Heatmap of viral reads (log2 scale) sequenced in 124 fecal samples collected from 35 women with type 1 diabetes (n=69 samples) and 26 without (n=55 samples) during pregnancy. Only viruses with ≥100 reads matched by BLAST at the species level were included and represented at the genus level. Number of viruses detected per specimen, frequency of each virus within the case or control group, and the mean log read counts are summarized by bar charts.

Figure 2. Longitudinal changes in the gut virome during pregnancy. Presence-absence heatmap of viruses detected over multiple trimesters of pregnancy (T1, T2 and T3) in women with type 1 diabetes (n=28 individuals) and without (n=21 individuals).

Figure 3. Viruses differentially abundant between the gut of women with and without type 1 diabetes during pregnancy. Volcano plot of viruses with ≥2-fold difference (marked by vertical dotted lines) in abundance between pregnant women with and without type 1 diabetes. Only
differences with false discovery rate below 5% (p<0.05) as determined by edgeR are represented.

Species A (EV-A) and B enteroviruses (EV-B) are marked in red and blue, respectively. All other viruses represented in grey.
Figure 1

The figure depicts a bar chart showing the number of virus species per sample, categorized into two groups: T1D and Without T1D. Each virus species is represented by a bar, with the height indicating the frequency and the color indicating the mean read count on a Log2 scale.

The chart includes virus species such as Alphapapillomavirus, Astrovirus, Bocaparvovirus, Calicivirus, Camoivirus, Chlorovirus, Circo_3nonclassified, Circovirus, Cootervirus, Enterovirus, Echovirus, Gyrovirus, Mamavirus, Marseille_3nonclassified, Marseillevirus, Mastrevirus, Minv_3nonclassified, Norovirus, Papilloma_3nonclassified, Parechovirus, Picobirnavirus, Prasinovirus, Protoparvovirus, Pyrmavirus, Salivirus, Sapovirus, Simpexivirus, and Tobamovirus.
Figure 2

| Participant | Test 1 | Test 2 | Test 3 | Test 4 | Test 5 | Test 6 | Test 7 | Test 8 | Test 9 | Test 10 | Test 11 | Test 12 | Test 13 | Test 14 | Test 15 | Test 16 | Test 17 | Test 18 | Test 19 | Test 20 | Test 21 | Test 22 | Test 23 | Test 24 | Test 25 | Test 26 | Test 27 | Test 28 | Test 29 | Test 30 | Test 31 | Test 32 | Test 33 | Test 34 | Test 35 | Test 36 | Test 37 | Test 38 | Test 39 | Test 40 | Test 41 | Test 42 | Test 43 | Test 44 | Test 45 | Test 46 | Test 47 | Test 48 | Test 49 | Test 50 | Test 51 | Test 52 | Test 53 | Test 54 | Test 55 | Test 56 | Test 57 | Test 58 | Test 59 | Test 60 | Test 61 | Test 62 | Test 63 | Test 64 | Test 65 | Test 66 | Test 67 | Test 68 | Test 69 | Test 70 | Test 71 | Test 72 | Test 73 | Test 74 | Test 75 | Test 76 | Test 77 | Test 78 | Test 79 | Test 80 | Test 81 | Test 82 | Test 83 | Test 84 | Test 85 | Test 86 | Test 87 | Test 88 | Test 89 | Test 90 | Test 91 | Test 92 | Test 93 | Test 94 | Test 95 | Test 96 | Test 97 | Test 98 | Test 99 | Test 100 | Test 101 | Test 102 | Test 103 | Test 104 | Test 105 | Test 106 | Test 107 | Test 108 | Test 109 | Test 110 | Test 111 | Test 112 | Test 113 | Test 114 | Test 115 | Test 116 | Test 117 | Test 118 | Test 119 | Test 120 | Test 121 | Test 122 | Test 123 | Test 124 | Test 125 | Test 126 | Test 127 | Test 128 | Test 129 | Test 130 | Test 131 | Test 132 | Test 133 | Test 134 | Test 135 | Test 136 | Test 137 | Test 138 | Test 139 | Test 140 | Test 141 | Test 142 | Test 143 | Test 144 | Test 145 | Test 146 | Test 147 | Test 148 | Test 149 | Test 150 | Test 151 | Test 152 | Test 153 | Test 154 | Test 155 | Test 156 | Test 157 | Test 158 | Test 159 | Test 160 | Test 161 | Test 162 | Test 163 | Test 164 | Test 165 | Test 166 | Test 167 | Test 168 | Test 169 | Test 170 | Test 171 | Test 172 | Test 173 | Test 174 | Test 175 | Test 176 | Test 177 | Test 178 | Test 179 | Test 180 | Test 181 | Test 182 | Test 183 | Test 184 | Test 185 | Test 186 | Test 187 | Test 188 | Test 189 | Test 190 | Test 191 | Test 192 | Test 193 | Test 194 | Test 195 | Test 196 | Test 197 | Test 198 | Test 199 | Test 200 |