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Analysis of mRNA Expression Levels in FFPE Testicular Biopsies Using Real-time PCR Arrays

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Abstract

Background and objective: Tissues archived for long-term storage as formalin-fixed, paraffin-embedded (FFPE) samples represent a rich source of material for genomic studies, but the nucleic acid isolation and downstream analysis is technically difficult. This is especially true when conducting mRNA expression studies, which strongly depend on the quality and quantity of the starting material for successful data normalization. Our objective was to investigate the mRNA expression levels in testicular FFPE samples using real-time PCR arrays and to present our experience with some technical challenges. Methods: Total RNA was extracted from FFPE samples from six patients with hypospermatogenesis and three controls using the Qiagen AllPrep DNA/RNA FFPE Kit. The integrity of the isolated RNA was assessed by an Agilent Bioanalyzer 2100. Qiagen Human Male Infertility RT² Profiler PCR Arrays were used to study the expression of 84 genes. Results: Our experience with the analysis of the FFPE testicular samples using the RT² Profiler PCR Array showed difficulties with the data normalization, despite using the same amount of starting material and similar values for the RNA integrity numbers (RINs) across all the samples, as recommended by the manufacturer. By using the percentage of the relatively intact RNA (area between 150 bp and 4,000 bp on the electropherograms of the Bioanalyzer 2100), we observed a negative correlation between amount of the intact RNA and average Ct values of the analyzed genes. Our initial results using PCR Array analysis on FFPE testicular tissues revealed 38 differentially expressed genes that are enriched in interactions, as well as for Gene Ontology molecular and cellular function terms. Conclusions: Stratification of the FFPE samples according to their percentage of intact RNA could improve the quantitative real-time PCR Array analysis.

Introduction

Infertility is a worldwide problem affecting 5–7% of the general male population, and in about 50% of the reported cases the main cause for male infertility is idiopathic.¹ In the majority of cases of idiopathic male infertility the causes might be of a genetic nature because of the vast number of genes and gene interactions involved in spermatogenesis and male fertility. Testicular biopsies archived as formalin-fixed, paraffin-embedded (FFPE) samples represent a valuable resource for the analysis of the influence of DNA sequence changes and RNA expression on spermatogenesis. However, the isolation and downstream analysis of nucleic acids from these samples are technically difficult due to chemical modifications and degradation associated with the fixation process and storage.² In particular, the presence of formaldehyde induces cross-linking between nucleic acids and proteins, and the low pH (<1) induces fragmentation.³,⁴ Nevertheless, FFPE-archived tissues are more easily accessible than fresh or frozen tissues, thus much effort has been made to use FFPE tissues for different genetic analysis.

One approach in the search for genetic causes of male infertility is to analyze testicular gene expression profiles of patients with idiopathic male infertility and perform a comparative analysis to the profiles of subjects with normal spermatogenesis. Several different methodologies are available for this, including northern blotting, reverse transcription-quantitative polymerase chain reaction (RT-qPCR), microarray analysis and RNA sequencing. Although RT-qPCR is considered as one of the gold-standard methods for detection and measurement of mRNA transcripts, it has a disadvantage since only a limited number of genes can be analyzed simultaneously. Recently, PCR Arrays have been introduced that combine the real-time PCR performance with the ability of arrays to detect the expression of many genes simultaneously. Such PCR Array methodology that employs SYBR® Green chemistry was successfully validated against TaqMan PCR, microarrays and other gene expression measurement technologies.⁵,⁶ However, these validation studies were performed on high quality RNA reference samples, the first one being from normal human brain tissue and the second one being a pool of 10 human cell lines. Studies using the PCR Arrays on FFPE tissues are scarce.
Here, we are reporting our experience with the analysis of gene expression of 84 genes required for normal male fertility in testicular biopsies archived as FFPE tissues from male infertile patients. Our goal was to address some possible technical challenges when working with low-quality RNA during gene expression studies and to present initial results of differentially-expressed genes in testicular tissues of male infertile patients with hypospermatogenesis.

Materials and methods

Samples

FFPE-archived tissues from three men diagnosed with normal spermatogenesis (patients with obstructive azoospermia) and six men diagnosed with hypospermatogenesis (infertile patients) based on histopathological findings were used in the study. While an ideal study design would have been to use biopsies from healthy fertile patients with normal testicular function as controls, in practice this is very hard to achieve; thus, as shown in other studies, testicular biopsies from men with obstructive azoospermia and histopathological finding of normal spermatogenesis were used as an acceptable alternative. This study was approved by the Ethical Committee of the Macedonian Academy of Sciences and Arts. All subjects gave written informed consent for participation, in accordance with the Declaration of Helsinki.

RNA isolation, quality control and performance of real-time qPCR array analysis

The total RNA was extracted using the AllPrep DNA/RNA FFPE Kit from Qiagen (Hilden, Germany), with the simultaneous isolation of DNA and RNA being performed after deparaffinization of the FFPE samples using xylene. We have previously compared this kit with the RNA isolation method using the Trizol reagent and with two other commercial kits for RNA isolation only; we had found that the AllPrep DNA/RNA FFPE Kit was best suited for our needs because the obtained RNA samples were of consistent quality and quantity that enabled us to perform miRNA microarray expression study. RNA quantity and purity were determined using the NanoDrop ND-2000 (NanoDrop Technologies, Wilmington, DE, USA), and RNA integrity (as RNA integrity number, RIN) was assessed using the RNA 6000 Nano Chip on a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). The isolated RNA was analyzed using the Human Male Infertility RT² Profiler PCR Array (Cat# PAHS-165ZC; Qiagen), which profiles the expression of 84 key gene transcripts detected in the male germline that are known to be involved in different fertility- and sperm-related processes in the cell, including spermatogenesis, fertilization, male sex differentiation, cell motility, cell cycle and response to stress. The cDNA synthesis, pre-amplification and real-time PCR analyses were conducted according to the instructions given by the manufacturer. A starting amount of 640 ng of total RNA (maximum amount available from the sample with the lowest RNA concentration) was used for all samples for the reverse transcription and qPCR. The real-time qPCR was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

Data analysis

Following the real-time qPCR, the data from all samples were analyzed using the same Ct threshold. The Ct threshold was set to an arbitrary value of 0.067761 after averaging all plates and performing manual inspection for best-fit, while the baseline was set to 3 cycles below the lowest Ct. Afterwards, the Ct values for each sample were exported into separate Microsoft Excel files for web-based RT² Profiler PCR Array Data Analysis 3.5 (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). Quantification of the relative changes of gene expression levels (fold-change) was performed using the $2^{-\Delta\Delta Ct}$ method. The p-values were calculated using a Student’s t-test (two-tail distribution and equal variances between the two samples) on the $2^{-\Delta Ct}$ values. The percentage of intact RNA, which was arbitrarily set between 150–4,000 base pairs (bp), was determined by 2100 Expert Software (Agilent Technologies). Pearson’s correlation analysis was performed using the Statistical Package for Social Sciences, Version 19 (SPSS, Chicago, IL, USA). The level of the two-tailed test of significance was set at $p<0.05$. Analysis for possible interactions between genes.
and the building of network interactions was carried out using STRING v10, while Gene Ontology (GO) analysis was performed using WebGestalt.9–11

Results

Normalization of gene expression between plates

The analysis of relative expression in the RT² profiler PCR Arrays was based on defining the same Ct threshold values across all runs and then performing normalization with stably expressed genes in all samples. Although the RIN numbers (which ranged between 2.1 and 2.5) and the total RNA input amount was similar in all samples, there were large differences in the expression levels of the five housekeeping genes in the nine studied samples (Fig. 1). There was no correlation between average Ct values of the five housekeeping genes and the RIN numbers in the nine analyzed samples.

In order to understand the discrepancies in the Ct values of the housekeeping genes between the samples, we determined the percentage of the relatively intact RNA (set between 150 bp and 4,000 bp) in each sample by analyzing the electropherograms (Fig. 2).
The percentages of the selected area varied between 40% and 71% in the different samples.

Control samples, from patients with normal spermatogenesis, are expected to have similar expression levels of all analyzed genes; hence, we averaged the Ct values of all 89 genes in the three controls and compared them with the area percentage of the intact RNA. There was a strong significant negative correlation between the averaged Ct values and the amount of intact RNA in the controls (Fig. 3A). The same comparison was made for the five housekeeping genes in all nine samples, since they are expected to be expressed at similar levels; the results showed a moderate significant negative correlation (Fig. 3B).

When analyzing the average Ct values of the five housekeeping genes in the group of patients with hypospermatogenesis, the correlation was found to be similarly moderate but without significance (r = -0.686, p = 0.1324). When analyzing the average Ct values of the 84 targeted genes in the group of patients with hypospermatogenesis, the correlation found to be weak and without significance (r = -0.246, p = 0.6383). This weak correlation might be due to the different severity of hypospermatogenesis in the patients that may have influenced the expression levels of the 84 analyzed genes.

**Analysis of differentially expressed genes**

According to the manufacturer’s protocol, normalization of the expression data of the analyzed samples can be performed using one of the housekeeping genes or any other of the 84 genes, provided that the Ct values of the gene used for normalization does not differ more than 1.5 cycles in all samples (plates). In our study, none of the housekeeping genes satisfied this criterion. However,
in six out of nine samples (plates) normalization was possible with the SOD2 gene (superoxide dismutase 2, mitochondrial), which plays a key role in the protection against oxidative stress in the mitochondria. The best strategy for choosing the right genes for data normalization, as previously shown, is to test the larger number of genes and to select those with lowest variability between the studied groups of samples. The SOD2 gene is expressed in various tissues, including different testicular cells, so we chose this gene for normalization in our mRNA expression studies of testicular biopsies. This might be a limitation of our study, since we cannot exclude the possibility that the SOD2 gene is differentially expressed between physiological and pathological conditions. The inclusion of experimentally-validated control genes, instead of the widely used housekeeping genes, might improve these commercially available PCR arrays.

When the fold-change cut-off was set at 4, a number of genes showed lower expression among the patients with hypospermatogenesis than in the patients (controls) with normal spermatogenesis. Furthermore, the genes involved in spermatogenesis and fertilization showed a higher number of down-regulated genes, as compared to genes involved in male sex differentiation, cell motility, cell cycle and response to stress (Fig. 4).

All 84 genes were down-regulated in the patients with hypospermatogenesis, but the difference was significant only for 38 of them. The fold-changes and p-values for all of the 84 analyzed genes for the six patients with hypospermatogenesis are given in Supplementary Table S1.

### Discussion

The analysis of gene expression using real-time PCR is a standard and widely used technique because of the wide dynamic range of quantification, high sensitivity, accuracy and robustness. The quantification of changes in gene expression could be absolute, using standard curves, or relative, using a calibrator gene whose expression level between samples is constant. In order to investigate the expression of many genes simultaneously, we used a RT2 Profiler PCR Array from Qiagen for simultaneous amplification and expression detection of 84 genes detected in the male germ line.

One of the recommendations for analysis using PCR Arrays is to use the same input amount of RNA and the same quality of RNA samples, as ascertained through RIN number, as well as the same Ct threshold between plates. Even though we fulfilled these requirements, our analysis of nine FFPE samples still showed high variation of Ct values between plates for the five pre-selected housekeeping genes. We found no correlation between RIN numbers and variation in Ct values.

After a visual inspection of BioAnalyzer electropherograms, we noticed differences between samples in the curve shapes, and for that reason we decided to investigate a possible correlation between the amount of relatively intact RNA (150–4,000 bp) and variation in Ct values. We found a strong negative correlation between the amount of intact RNA and the average Ct values of all 89 genes in the three control samples. Furthermore, a moderate negative correlation was observed when we investigated five housekeeping genes in all samples. Fragments with sizes between 70–150 bp, even though abundant, could not contain one of the primer sequences required for initial PCR amplification. Consequently, the samples with same RNA concentration but different percentage of intact RNA will generate different Ct values in a PCR reaction.

In the majority of studies of the RNA expression levels in FFPE tissue samples, the RIN number has been used as a primary measurement for RNA integrity. This applies not only for real-time PCR-based studies, as in our case, but also for microarray genome-wide expression studies. We propose methodology that takes into account not only the integrity of the RNA as a whole, but also the percentage of the relatively intact RNA between samples. In heavily-degraded samples, differences in this percentage could have a significant impact on the final result.

### Conclusion

Data normalization of the relative gene expression levels using a reference gene with stable expression across various conditions is an essential step in real-time quantitative PCR analysis. However, in samples with degraded RNA, especially for RNA from FFPE tissues which is often heavily degraded, erroneous results are possible due to different concentrations of intact RNA molecules in the different samples. Our experience with analysis of FFPE testicular samples using the RT2 Profiler PCR Array from Qiagen showed difficulties with data normalization, despite using the same amount of starting material and similar values for the RIN numbers across all the samples, as recommended by the manufacturer. We detected high correlation between the amount of intact RNA and the average Ct values of analyzed genes in the control samples.

Stratification of the FFPE samples according to their percentage of intact RNA could improve the quantitative real-time PCR Array analysis.

### Prospective

Simultaneous analysis of RNA expression levels of a large number of genes is a powerful tool for discovering the affected biological pathways and underlying genetic causes of complex conditions, such as male infertility. Targeted analysis of the genes previously found to be primarily expressed in the testicular tissue and related to the spermatogenesis process could be a cost-effective and robust method for analysis of a larger number of samples. However, design of such panels of genes should be accompanied with adequate solutions to overcome common technical difficulties, such as experimental validation of housekeeping genes, and especially addressing and improving the data analysis of samples from which RNA with sufficient quality is difficult to obtain.

Our initial results using PCR Array analysis on FFPE testicular tissues revealed 38 differentially-expressed genes. The network analysis for possible interactions between these 38 selected genes, using the STRING web tool showed that the resulting network is enriched in interactions ($p=1.73E−14$) (Fig. 5).

GO enrichment analysis for molecular function showed a significant enrichment in a subset of genes involved in nucleic acid binding (GO: 0003676), transcription cofactor activity (GO: 0003712), translation activator activity (GO: 0008494) and histone acetyl-lysine binding (GO: 0070577). For the cellular component, the analysis showed a significant enrichment in a subset of genes for the chromosomal portion (GO: 0044427), the microtubule-based flagellum (GO: 0009434), the CatSper complex (GO: 0036128) and a chromatoid body (GO: 0033391).

Further analyses on a larger number of samples are warranted to confirm our initial findings on the affected biological pathways in testicular tissues of patients with hypospermatogenesis.
Acknowledgements

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Designed the study (DPK), Performed the experiments (PN, KPI), Analyzed the data (PN, DPK), Contributed reagents/materials/analysis tools (KKS, VF, SL, TP, DPK), Wrote the manuscript (PN, DPK).

Supplementary information

(Supplementary information is linked to the online version of the paper on the Exploratory Research and Hypothesis in Medicine website.)

References


Diagnostic Evaluation of Anti-Tissue Transglutaminase and Anti-Endomysial Antibodies for Diagnosis of Coeliac Disease: Is There a Need for Duodenal Biopsy?

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Abstract

Background and Objectives: Although duodenal biopsy is suggested as the gold standard method for diagnosis of coeliac disease (CD), high levels of immunoglobulin A anti-tissue transglutaminase (IgA anti-TTG) followed by positivity for immunoglobulin A anti-endomysial (IgA anti-EMA) have been used widely for the diagnosis. In this study, we tested the hypothesis that IgA anti-TTG and IgA anti-EMA tests are useful for diagnosis of CD, without need for endoscopy and duodenal biopsy. Methods: CD diagnosis was made with IgA anti-TTG >10 U/mL and IgA anti-EMA positivity in presence of villous atrophy. Receiver operating characteristic (ROC) analysis was performed to establish the cut-off values of the IgA anti-TTG that could predict the presence of IgA anti-EMA positivity and villous atrophy from histology results. Results: The cut-off values for IgA anti-TTG that predict positivity for IgA anti-EMA (sensitivity: 89.7%; specificity: 82%; positive predictive value: 92.4%) and villous atrophy (sensitivity: 88.9%; specificity: 78.5%; positive predictive value: 92.9%) were 17 and 30 U/mL, respectively. Conclusions: Serological testing can be used with high sensitivity and specificity for diagnosis of CD, without biopsy and histology. This significantly improves the timeliness and effectiveness of CD diagnosis.

Introduction

Coeliac disease (CD) is a chronic gastrointestinal disorder and is the most common immuno-mediates enteropathy, reportedly affecting about 1% of the populations in Western countries. The purported causes of CD include both environmental (gluten) and genetic (human leucocyte antigen, DQ2/DQ8) factors.1 In CD, ingestion of gluten, a protein commonly found in wheat, rye and barley is believed to activate the immune response, with the consequent of intestinal damage and villous atrophy. In addition, CD is characterized by a variety of intestinal and extra-intestinal manifestations related to the subsequent deficiency of macronutrients and micronutrients related to the disease condition.2–4

The most frequently reported clinical signs and symptoms of classical CD include malabsorption (diarrhoea, steatorrhoea, unintentional weight loss) and of non-classical CD include anaemia, hepatic steatosis, osteopenia/osteoporosis and mouth ulcer.5 Untreated CD can lead to various complications that can severely affect quality of life, such as nutritional deficiencies, anaemia, osteoporosis and growth failure, as well as the possible development of other autoimmune disorders and malignancies.6–8 In current clinical practice, diagnosis of CD is normally carried out by first-line serology testing, with results of immunoglobulin A anti-tissue transglutaminase (IgA anti-TTG; high) and immunoglobulin A anti-endomysial (IgA anti-EMA; positive). Patients with CD-indicative serology test results are then usually referred for duodenal biopsy to confirm the intestinal damage and villous atrophy.6–7

Although the duodenal biopsy and histology investigation has been considered as the gold standard for diagnosis of CD, there are limitations to this approach; these include availability, unpleasantness, invasiveness, artefacts due to a non-longitudinal cut, poor specificity and high cost.9–10 For diagnosis of CD, there are still questions and challenges remaining, particularly regarding who should be screened, how to screen, how to properly interpret the serologic test results and whether there is a need for further endoscopy, duodenal biopsy and/or histology investigation. In patients with suspected CD, the IgA anti-TTG test is most commonly used as the first choice test to detect the presence of antibodies, but there are also questions about its accuracy. If there is a strong and ongoing clinical suspicion of CD, IgA anti-EMA should be requested. However, there is still limited information regarding the sensitivity and specificity of IgA anti-TTG and IgA anti-EMA tests. A clearer understanding of the sensitivity and specificity of IgA anti-TTG and IgA anti-EMA will allow clinicians to better interpret test results and make a timelier and better informed diagnosis, ultimately supporting better administration of subsequent treatment. There are a number of studies suggesting that IgA anti-TTG levels are significantly and positively correlated with the severity of intestinal damage, but there are questions that remain as to whether there is a need for duodenal biopsies and histology investigations for all patients who present with positive serology results.10–15

In this study, we sought to determine the cut-off values for IgA anti-TTG to predict positivity for IgA anti-EMA and the presence of villous atrophy and we tested the hypothesis that high sensitivity and specificity of IgA anti-TTG and IgA anti-EMA would exclude...
the need for an endoscopy and histology procedures for diagnosis of CD in adults.

**Methods**

**Study population**

A total of 13,086 consecutive adult patients (>18 years) referred from primary care from January 2012 to May 2016 with suspected CD according to presence of diarrhoea, steatorrhoea, unintentional weight loss, abdominal pain, bloating, anaemia, osteopenia/osteoporosis, hepatic steatosis, mouth ulcer and family history of CD were considered for study inclusion. All subjects were tested for the first time for IgA anti-TTG. Patients with IgA anti-TTG >10 U/mL were then tested for IgA anti-EMA; none of these patients were on the gluten-free diet. Patients with positivity for both IgA anti-TTG and IgA anti-EMA underwent subsequent endoscopy with duodenal biopsies/histology. CD diagnosis was made according to the presence of villous atrophy associated with anti-TTG IgA >10 U/mL and positivity for IgA anti-EMA.1

**Serology**

Serum anti-TTG IgA was analysed by immunoassay (Phadia 250; Thermo Fisher Scientific) and analysis of serum IgA anti-EMA was based on the fluorescein-labelled anti-human IgA (monkey oesophagus slides; BioSystems) and visualized with the aid of a fluorescence microscope, by immunofluorescence.

**Endoscopy and histology**

Multiple endoscopic duodenal biopsies were obtained by oesophagogastroduodenoscopy. Analysis of the duodenal biopsies was carried out at the Department of Histopathology. CD was diagnosed by pathological changes of the small intestine that included intraepithelial lymphocytosis, crypt hyperplasia and subtotal or total villous atrophy.9

**Statistical analysis**

Receiver operating characteristic (ROC) analysis was used to determine the cut-off values of IgA anti-TTG to predict positivity for IgA anti-EMA and villous atrophy by assessing the area under the curve (AUC) in addition to the sensitivity, specificity, positive predictive and negative predictive values. The ROC curves were constructed by plotting the sensitivity (true-positive) on the ordinate as a function of the complement of specificity (false-positive) for all possible cut-off values of the IgA anti-TTG test. Greater deviation towards the left upper corner with a high and significant AUC indicated good prediction of positive IgA anti-EMA and villous atrophy. All statistical inferences were made based on a two-sided significant level of $P<0.05$ and were performed using IBM® SPSS® Statistics version 21.0.

**Results**

Demographic characteristics, clinical symptoms, and serology and histology results of the patients with positivity for IgA anti-TTG are shown in Table 1. During the study period, 13,086 subjects were referred for suspected CD and 166 were found to be IgA anti-TTG-positive (1.3%). Out of those 166 patients, 136 (82%) were IgA anti-TTG-positive and 142 (85.5%) had villous atrophy. The mean IgA anti-TTG titre was 70.0±59.7 U/mL. The prevalence of positive IgA anti-TTG was significantly higher in females (73.4%) than males (26.5%). The most common symptoms and conditions associated with positive IgA anti-TTG and CD were abdominal pain and bloating (26.5%), anaemia (21.7%) and diarrhoea (22.3%).

ROC analysis showed that IgA anti-TTG with cut-off values of 17 U/mL (AUC=0.814 CI: 0.722–0.895, $p<0.001$) predicts positivity for IgA anti-EMA (Fig. 1) with a high sensitivity (89.7%), specificity (82.7%), positive predictive value (92.4%) and positive likelihood ratio (2.6; 1.6–4.3, $p=0.001$), as shown in Table 2. In addition, results from ROC analysis showed that IgA anti-TTG with a cut-off value of 30 U/mL (AUC=0.715 CI: 0.580–0.850, $p=0.003$) predicts villous atrophy (Fig. 2) with a high sensitivity (88.9%), specificity (78.5%), positive predictive value (92.9%) and positive likelihood ratio (2.6; 1.0–4.1, $p=0.01$), as shown in Table 2.

**Discussion**

In this study, the diagnostic evaluation of serology tests for the diagnosis of CD was investigated and we also tested the hypothesis that serology tests are accurate and sensitive enough to allow for avoidance of intestinal biopsy and histology investigation with its limitations and cost. Histological examination is still considered as the gold standard method for diagnosis of CD in adults. However, there are limitations in endoscopy and histology procedures, including invasiveness, high costs and poor specificity in cases of duodenal lymphocytosis9, as some histological patterns are also seen in other clinical conditions.9 In this study, the major clinical
conditions that were associated with positive IgA anti-TTG were abdominal pain, bloating, anaemia and diarrhoea, with other minor conditions recorded, including weight loss, osteoporosis/osteopenia, diabetes, hepatic osteitis and mouth ulcer. There are some clinical conditions, such as liver disease, rheumatoid arthritis and inflammatory bowel disease, that may be associated with high IgA anti-TTG and can lead to false positive findings for CD. However, with positivity for IgA anti-EMA accompanied by symptoms and risk factors associated with CD, it is more likely that the patients are suffering from CD.

ROC analysis showed that IgA anti-TTG levels at 17 U/mL predicted positivity for IgA anti-EMA, and at 30 U/mL predicted villous atrophy with high sensitivity, specificity and positive predictive values. Results from this study are consistent with the previous studies that have suggested a strong relationship between IgA anti-TTG levels and small intestine histopathology and being highly specific for villous atrophy; thus, duodenal biopsy may not be required and may be avoided when IgA anti-TTG levels are 3–5 times greater than normal levels. This endoscopy-free diagnostic approach to CD could be particularly useful in situations where endoscopy is not available and where performing endoscopy would be very difficult, such as in patients who are elderly, pregnant, afflicted with Down’s syndrome or mental illness. There are additional adult and children patients who may be unable or unwilling to undergo an endoscopy. Under these circumstances, assessment of the serological assay showing high IgA anti-TTG and positivity for IgA anti-EMA can have a supportive role.

Table 2. Diagnostic evaluation of IgA anti-TTG test for the prediction of positivity for IgA anti-EMA and villous atrophy

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<tr>
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<th>IgA anti-EMA</th>
<th>Villous atrophy</th>
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<tr>
<td>AUC</td>
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<td>0.715</td>
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<td>95% CI</td>
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<td>0.580–0.850</td>
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<td>Specificity</td>
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<td>Positive predictive value</td>
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<td>Negative likelihood ratio</td>
<td>0.16 (0.09–0.28)</td>
<td>0.35 (0.19–0.64)</td>
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</table>
North Devon, UK. Results from this study suggest that the use of a diagnostic approach based on serology results showing IgA anti-TTG levels of 17 U/mL to predict IgA anti-EMA and 30 U/mL to predict villous atrophy. Duodenal biopsy may not be required and could be avoided if IgA anti-TTG levels are 3–5 times greater than normal levels. This could have an impact on timely diagnosis of CD and significant reduction in CD diagnosis-related costs, as has been reported from previous studies and supports a cost-sparing and biopsy-free approach for diagnosis of CD.19

In conclusion, in adult patients with positivity for both IgA anti-EMA and IgA anti-TTG, the latter at a level of 30 U/mL, a diagnosis of CD could be reached without endoscopy and subsequent biopsy/histology. These results could contribute to improving the diagnostic work-up of CD for a significant reduction in diagnosis-related costs.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Acquisition of data (TW, HM, LM, RB, MS), analysis and interpretation of data (JG, JO), drafting of manuscript (JG), critical revision (JO).

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Avian Influenza in Wild Birds from South America: Review, Implications and Perspectives

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Abstract

Avian influenza viruses (AIVs) have been recorded in a broad variety of hosts, including humans, terrestrial and marine mammals, and domestic and wild birds. Wild aquatic birds are recognized as the chief natural reservoirs of AIV, and their migratory flyways can serve as routes for the dispersion of the virus across countries and continents. Although AIV is one of the most studied pathogens in the world, studies on the ecology and epidemiology of this virus in South America are few and fragmented. In this review, we examine the current state of the art on the epidemiology of AIV in wild birds in South America. Current evidence corroborates that many of the broad epidemiological patterns that have been documented in other continents, such as the role played by Anseriformes and Charadriiformes in the maintenance and spread of AIV, are also true in South America. On the other hand, the fact that AIV prevalence in South American studies appears to be remarkably lower than that observed in other continents, along with the presence of endemic taxa of birds that may be highly susceptible to AIV, indicates that South America may have distinct characteristics that modulate the epidemiology of AIV in unique ways. However, our knowledge on the occurrence of AIV in South America is still limited and there are important gaps in the species and geographic distribution of the sampling effort.

Introduction

Avian influenza viruses (AIVs) are members of the genus Influenzavirus A (Orthomyxoviridae) and contain 8 negative-sense RNA segments. These viruses are classified in subtypes based on the antigenic properties of the two glycoproteins expressed on the viral envelope, hemagglutinin (HA) and neuraminidase (NA). To date, 16 HA subtypes (H1-H16) and 9 NA subtypes (N1-N9) have been described in birds and can be found in multiple combinations.1,2 Novel HA and NA subtypes (H17-H18 and N10-N11) were recently described in Influenzavirus A from bats in South America, however current evidence indicates these subtypes might not be able to infect birds even though they can infect human and canine cells in vitro.3–5

The first suspected human cases of influenza date back to the writings of Hippocrates in 410 B.C., and since then a number of epidemics and 11 pandemics have occurred.6,7 The most devastating was the “Spanish flu”, an H1N1 pandemic that killed approximately 50 million people in 1918.8,9 More recently, the H1N1 pandemic of 2009 claimed the lives of 284,500 people.10 AIVs have been recorded in a broad variety of hosts, including humans, terrestrial and marine mammals, and domestic and wild birds. Aquatic birds are recognized as the chief natural reservoirs of AIV, especially those from the orders Anseriformes (e.g. teals, ducks, geese and swans) and Charadriiformes (e.g. shorebirds, gulls and terns).11,12 Most AIV infections in these avian taxa are subclinical or accompanied by only mild clinical signs, and as a result they are effective reservoirs of infection and play a central role in the ecology and evolution of AIV.11–14 Studies in North America have shown that Anseriformes are central to the long-term persistence of AIV, while migratory Charadriiformes play a role in the short-term dissemination of the virus over long distances.15 Furthermore, because birds do not recognize geopolitical borders, their migratory flyways can serve as routes for the dispersion of the virus across countries and continents.11

While the ecology of AIV has been extensively studied in North America, much less information is available for South America. There are major flyways connecting South and North America, whereas migratory routes connecting South America to other continents are not as common.13,16 It is, therefore, reasonable to expect that the genetics and ecology of AIV in South America resembles those of the virus in North America. However, because North and South America differ substantially in terms of their avian communities, ecosystems and environmental conditions, differences in ecology and epidemiology may also be expected to occur.

In this review, we examine the current state of the art on the epidemiology of AIV in wild birds in South America, evaluating how the available information compares to what is known about the virus in North America and elsewhere, discussing the implications for public health, the poultry industry and wildlife conservation, and identifying limitations of and gaps in the knowledge to be addressed in future studies.

Early studies in South America

Most studies investigating the occurrence of AIV in wild birds

Keywords: Birds; Wildlife; Avian influenza virus; Epidemiology; Zoonosis; Public health.

Abbreviations: AIV, avian influenza virus; HA, hemagglutinin; HI, hemagglutination inhibition; HPAI, high pathogenicity avian influenza; LPAI, low pathogenicity avian influenza; NA, neuraminidase; rRT-PCR, real-time reverse transcription polymerase chain reaction.

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in South America were conducted in the last decade, with only a small number of studies conducted prior to 2005, all of them in Brazil. These early studies employed methods that are either no longer used in recent studies, such as viral isolation in cell cultures or serological testing for which there are no recent studies for comparison, or obtained atypical results that merit to be considered separately.17–19

The first studies on the occurrence of AIV in South America were conducted by Couceiro et al., who collected samples from 107 white-faced whistling-ducks (Dendrocygna viduata) and 39 ornamental birds in Rio de Janeiro from 1978 to 1979, and isolated AIV from 13 (12.1%) and 5 (17.2%) samples, respectively.20,21

The strains were isolated following inoculation in embryonated eggs, and were confirmed as AIV by double immunodiffusion. Hemagglutination inhibition (HI) revealed that the strains were inhibited by sera against subtype H6 or, for one strain, against subtype H7. The strains were isolated following inoculation in embryonated eggs, and were confirmed as AIV by double immunodiffusion. Hemagglutination inhibition (HI) revealed that the strains were inhibited by sera against subtype H6 or, for one strain, against subtype H7.

Research on AIV in wild birds in Brazil would be continued only two decades later, in 1997–1998, when Kawamoto et al. sampled 37 wild birds in the São Paulo state.17 In that study, AIV was isolated in cell culture (MDCK and NCIH 292) from 15 birds (40.5%), corresponding to 37.5% of the ruddy ground-ducks (Columbina talpacoti, n=8) and all the red-eyed vireos (Vireo olivaceus, n=3), red-cowled cardinals (Paroaria dominicana, n=2), double-collared seedeaters (Sporophila caerulescens, n=1) and lined seedeaters (Sporophila lineola, n=1) sampled in that study. The presence of AIV was confirmed by HI testing and real-time reverse transcription polymerase chain reaction (RT-PCR), and all strains were later characterized as H3N2.22,23

On the other hand, Soares et al. used the same methods but did not detect AIV in 86 ducks (Anatidae) captive at the São Paulo zoo.22

In 2004, the Brazilian Ministry of Health conducted two surveys for influenza virus in wild birds. The first study examined wild birds of Galinhos, Rio Grande do Norte state.24 A total of 381 Charadriiformes (Charadriidae and Scolopacidae) and 7 ground-ducks (Columbina sp.) were sampled, and AIV isolation in embryonated eggs was attempted. Because samples were pooled for testing, the apparent prevalence could not be clearly established. However, the pool of ground-dove samples and 12 of 21 pools (57.1%) of shorebird samples showed positivity to AIV, and HI testing indicated all isolates corresponded to subtype H3. The second study employed similar methods to test wild birds of Lagoa do Peixe, Rio Grande do Sul state, comprising: 169 Charadriiformes (Charadriidae, Laridae, Recurvirostridae, Rynchopidae, Scolopacidae), two Passeriformes and one Podicipediformes.25

Four of seven pools (57.1%) of shorebird samples showed positivity to AIV, and HI testing indicated two isolates corresponded to subtype H2 and the other two corresponded to subtype H4.

It is significant that these early studies identified percentages of positive samples that were significantly higher than those usually recorded in studies from other continents and in later studies conducted in South America.11 Possible explanations to such unusually high prevalence include suboptimal specificity of early diagnostic methods, field or laboratory contaminations, or the possibility that the samples obtained represented epizootic or otherwise unusual epidemiological conditions.

Therefore, because these early studies are in contrast with the findings of recent studies and might not faithfully represent the epidemiology of AIV in wild birds in South America, the reader of this review will address only studies that have been published in the last decade, from 2006 to 2016 (inclusive).

**Recent studies of AIV in South America**

Eighteen studies investigating the occurrence of AIV in wild birds in South America were published from 2006 to 2016 (Supplementary File S1). The publication of these studies did not follow an evident historical trend, with an irregular number of publications and sampling effort from year to year (Fig. 1). Such lack of a constant or gradual increase in the number of publications or sample size is likely a reflection of the fact that most studies were conducted as short-term research efforts of small academic groups rather than as long-term governmental or multi-institutional AIV surveillance programs.

From 2006 to 2016, most publications investigated AIV in wild birds sampled in Brazil, Argentina and Peru (Table 1). From a total of 19,755 samples tested in the continent during this period, 17,389 samples (88%) had been collected in these three countries, illustrating the strong bias in the sampling effort. When the accumulated sample sizes are contrasted with the area and number of
bird species of each country, it becomes evident that their distribution is not proportional to the territory of each country nor is it a fair representation of their avian diversity.26,27 In fact, nearly half of the countries of South America lack published studies on the occurrence of AIV in their wild birds. For instance, Colombia ranks first in avian diversity in the world, with 1,923 known bird species, and yet only one such study has been published to date. Similarly, Ecuador and Venezuela are also among the top five countries with highest avian diversity in South America, with respectively 1,675 and 1,405 bird species, and yet have not been the subject of publications on the occurrence of AIV in their wild birds. To place this in perspective, the United States and Canada have respectively 1,146 and 690 bird species.27

The distribution of AIV studies is uneven in relation to the taxonomical orders of the wild birds that were studied (Table 2). Because several publications fail to provide details on the number of individuals sampled for each avian species, it is not possible to determine the taxonomical order of the birds from which 7,018 samples were obtained (35.5% of the sampling effort). For the remaining samples, the orders Anseriformes and Charadriiformes were the most frequently targeted species, corresponding respectively to 48.3% and 29.1% of the samples tested for AIV. Smaller but still significant numbers of samples (>500) were evaluated for the orders Pelecaniformes (6.4%), Sphenisciformes (6.3%) and Suliformes (5.4%), all of which comprise aquatic species of birds.

The emphasis in Anseriformes and Charadriiformes is not surprising considering that these orders of aquatic birds are well known for their central role in the epidemiology of AIV in other continents.11,28 However, because the avian communities of South America are vastly different from those of the Northern Hemisphere, caution is advised not to assume that AIV epidemiology in the Neotropics will be identical to that observed in other well-studied continents. Other aquatic birds that may live in close proximity with Anseriformes and Charadriiformes should be investigated as they could potentially be involved in the maintenance and transmission of AIV. For instance, recent studies have found that penguins (Sphenisciformes) play an important role along with Charadriiformes in the persistence and evolution of AIV in Antarctica, a dynamic that could not have been witnessed in studies conducted in the Northern Hemisphere since penguins do not occur there.29,30 Inversely, murres and auklets (Alcidae) play an important role in AIV transmission in the Northern Hemisphere but are absent in the Southern Hemisphere.31,32

Furthermore, even though it is reasonable to consider aquatic birds as prime candidates for AIV surveillance in South America, the role of terrestrial birds as reservoirs of AIV should not be dismissed. It is worth highlighting that Alvarez et al. documented an outbreak of AIV subtype H1N1 in wild red-winged tinamous (Rhynchotus rufescens).33 This record corresponded to the first record of AIV in Tinamiformes, an order of non-migratory ground-dwelling birds that is endemic to Central and South America, which illustrates the need to investigate the occurrence of AIV in taxonomical groups that would not traditionally be expected to play a significant role in the epidemiology of these viruses.

When the geographic distribution of the sampling effort is examined in relation to the ecoregions of South America (Fig. 2, Supplementary File S1), another limitation of our current knowledge of AIV epidemiology in South America becomes evident. Of the 109 terrestrial ecoregions of continental South America (as classified by Olson et al.), to date only 16 ecoregions (14.7%) had wild birds tested for AIV.34 Because these ecoregions have distinct avian assemblages and environmental conditions, the prevalence and epidemiology of AIV may differ considerably among these areas, and comparative epidemiological studies have yet to be conducted.

Less than 0.1% of the AIV strains for which there are gene sequences in publicly-available databases correspond to viruses identified in avian hosts from South America.29,35 Unsurprisingly, the number of AIV isolates from South American wild birds has been historically correlated to the sampling effort accumulated by different studies (Fig. 3). This corroborates the interpretation that the small number of isolates is related to the proportionally smaller sampling effort that has been historically dedicated to the

### Table 1. Summary of the number of publications, sampling effort and AIV isolates from wild birds in South America from 2006 to 2016, by country

<table>
<thead>
<tr>
<th>Country</th>
<th>Area (km²)</th>
<th>Bird species</th>
<th>Publications</th>
<th>Sample size</th>
<th>Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argentina</td>
<td>2,780,400</td>
<td>1,035</td>
<td>3</td>
<td>7,504</td>
<td>10</td>
</tr>
<tr>
<td>Bolivia</td>
<td>1,098,580</td>
<td>1,425</td>
<td>2</td>
<td>93</td>
<td>1</td>
</tr>
<tr>
<td>Brazil</td>
<td>8,515,767</td>
<td>1,832</td>
<td>8</td>
<td>2,998</td>
<td>7</td>
</tr>
<tr>
<td>Chile</td>
<td>756,950</td>
<td>527</td>
<td>2</td>
<td>79</td>
<td>5</td>
</tr>
<tr>
<td>Colombia</td>
<td>1,197,411</td>
<td>1,923</td>
<td>1</td>
<td>2,013</td>
<td>2</td>
</tr>
<tr>
<td>Ecuador</td>
<td>283,560</td>
<td>1,675</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>French Guiana</td>
<td>91,000</td>
<td>727</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Guyana</td>
<td>214,970</td>
<td>796</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Paraguay</td>
<td>406,750</td>
<td>719</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Peru</td>
<td>1,285,220</td>
<td>1,828</td>
<td>3</td>
<td>6,887</td>
<td>31</td>
</tr>
<tr>
<td>Suriname</td>
<td>163,270</td>
<td>728</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Uruguay</td>
<td>176,220</td>
<td>470</td>
<td>1</td>
<td>181</td>
<td>0</td>
</tr>
<tr>
<td>Venezuela</td>
<td>912,050</td>
<td>1,405</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>17,882,148</td>
<td>3,505</td>
<td>18</td>
<td>19,755</td>
<td>56</td>
</tr>
</tbody>
</table>

The area and numbers of bird species of each country were obtained respectively from UNSD and Lepage.26,27 Numbers of publications from different countries overlap because one publication provides information relating to multiple countries.50
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Continent. However, even when this limitation is taken into account, current evidence also suggests that AIV prevalence might be relatively lower in South America than in North America and elsewhere. In a global review, Olsen et al. found that AIV prevalence was 7.7% for Anseriformes (n=44,318) and 1.2% for Charadriiformes (n=19,663). Similar levels of prevalence were consistently found in regional studies in North America. In comparison, studies in South America have found an overall prevalence of 0.21% in Anseriformes (range: 0.25–0.86%) and 0.32% in Charadriiformes (range: 0.21–3.80%) (Table 3). It is worth noting that Mathieu et al. stands out among South American studies for having found a slightly higher AIV prevalence (3.8%), which is probably related to the fact that this study investigated gulls that had been found dead, as opposed to actively capturing healthy birds in their natural habitat.

The reasons as to why South American wild birds appear to have a lower prevalence of AIV are unclear, and future studies will be necessary to confirm whether this is a widespread pattern and identify possible causes, or to dismiss the possibility that this reflects differences in sample collection season or storage. A possible explanation is that South America has a relatively lower diversity of Anseriformes (North America has 74 spp. and South America has 58 spp., of which 26 spp. are shared) and Charadriiformes (North America has 185 spp. and South America has 147 spp., of which 109 species are shared). Another factor at play may be the differences in families and subfamilies that each continent harbors. For Anseriformes, North America has more species in the subfamilies Anserinae (20 spp. and 3 spp. in North and South America, respectively) and Merginae (18 spp. and 3 spp.) and less species in the subfamily Tadorninae (1 sp. and 7 spp.) and species for which the taxonomy is unresolved (3 spp. and 9 spp.); species of the Anatinae subfamily are equally represented in both continents (18 spp. and 20 spp.). For Charadriiformes, the main difference that stands

<table>
<thead>
<tr>
<th>Taxonomic order</th>
<th>Argentina</th>
<th>Bolivia</th>
<th>Brazil</th>
<th>Chile</th>
<th>Colombia</th>
<th>Peru</th>
<th>Uruguay</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accipitriformes (68 spp.)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Caprimulgiformes (333 spp.)</td>
<td>3</td>
<td>4</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cariamiformes (2 spp.)</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Ciconiiformes (3 spp.)</td>
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<td>1</td>
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<td>14</td>
<td>29</td>
<td>43</td>
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<tr>
<td>Coraciiformes (16 spp.)</td>
<td>2</td>
<td>11</td>
<td>13</td>
<td></td>
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<td>Cuculiformes (23 spp.)</td>
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<td>Eurypygiformes (1 sp.)</td>
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<td>Galbuliformes (54 spp.)</td>
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<td></td>
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<tr>
<td>Gruiformes (56 spp.)</td>
<td>22</td>
<td>100</td>
<td>122</td>
<td></td>
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<td></td>
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<tr>
<td>Opisthocomiformes (1 sp.)</td>
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<td></td>
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<tr>
<td>Passeriformes (2024 spp.)</td>
<td>45</td>
<td>195</td>
<td>31</td>
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<td></td>
<td></td>
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<tr>
<td>Pelecaniformes (44 spp.)</td>
<td>8</td>
<td>3</td>
<td>343</td>
<td>461 [1]</td>
<td>815 [1]</td>
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<tr>
<td>Piciformes (134 spp.)</td>
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<td>1</td>
<td></td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>Podicipediformes (10 spp.)</td>
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<tr>
<td>Procellariiformes (92 spp.)</td>
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<td>19</td>
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<tr>
<td>Psitaciformes (131 spp.)</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rheiformes (2 spp.)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sphenisciformes (17 spp.)</td>
<td>806</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strigiformes (45 spp.)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Suliformes (23 spp.)</td>
<td>304</td>
<td>382</td>
<td>686</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tinamiformes (45 spp.)</td>
<td>77 [1]</td>
<td>77 [1]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trogoniformes (18 spp.)</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Numbers within brackets indicate the number of AIV strains isolated and/or confirmed through gene sequencing. The number of species recorded in South America for each order was obtained from Lepage.27
out is that the Alcidae family, a group that is well documented for carrying AIV, is represented by 23 spp. in North America while being absent from South America.27,31,32

AIV isolates and subtypes

Studies published from 2006 to 2016 have produced 56 AIV isolates from wild birds in South America (Supplementary File S2). The subtype of HA and NA could not be determined for one and four isolates, respectively. The remaining isolates represent 23 antigen combinations (Table 4). The most frequent HA subtypes identified to date are H13 (18.2%), H3 (14.5%) and H6 (12.7%), whereas N2 (28.8%) and N9 (26.9%) are the most frequent NA subtypes. Only HA subtypes H8, H14, H15 and H16 and NA subtype N4 have yet to be recorded in wild birds in South America; this is unsurprising as these subtypes are generally rare, with the exception of N4 in Charadriiformes.13

The distribution of HA and NA subtypes among Anseriformes and Charadriiformes is generally similar in South and North America (based on data summarized by Krauss et al.) (Fig. 4).13 However, there are a few exceptions. It may be noted that South American AIV strains had a relatively higher frequency of subtypes H13 and N1 in Charadriiformes, N2 in Anseriformes, and N9 in both Anseriformes and Charadriiformes. On the other hand, while N6 is relatively frequent in Anseriformes from North America, as are H9 and N4 in Charadriiformes, these subtypes have yet to be recorded in those avian orders in South America. The reasons for these discrepancies are not known, and while they could reflect actual differences in AIV epidemiology they could also be
related to differences in the distribution of the sampling effort in the different species/families within each order. For instance, it is well established that in North America the subtype H13 is more frequently found in gulls than in other Charadriiformes, a pattern that also seems to occur in South America (70% of the H13 strains recorded for this continent had been obtained from gulls).\cite{46,47} It is, therefore, reasonable to suspect that the higher percentage of H13 isolates in South America than in North America may reflect a greater proportion of gulls having been sampled in South America.

All H5 or H7 strains isolated in South America to date were found to have low pathogenicity, as determined by sequence analysis of the HA cleavage site and/or inoculation experiments.\cite{37,38,48-51} However, the H7N3 strain identified from cinnamon teals (\textit{Anas cyanoptera}) by Spackman \textit{et al.} merits further consideration.\cite{48,49} This strain had an HA cleavage site sequence indicative of low pathogenicity, and infection experiments corroborated its low pathogenicity to chickens and turkeys. However, its HA gene sequence was virtually identical to that of a highly pathogenic H7N3 strain identified in chickens in Chile 1 year later, suggesting that a genetic shift occurred with the insertion of 30 nucleotides at the HA cleavage site. This case, therefore, corroborates that low pathogenicity strains circulating in wild birds can undergo genetic

![Fig. 3. Relationship between the published sample size and number of AIV strains from wild birds in South America from 2006 to 2016. Each dot represents the state of knowledge at the time of publication of a study, which were included in chronological order of publication.](image)

<table>
<thead>
<tr>
<th>Country and references</th>
<th>Anseriformes</th>
<th>Charadriiformes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Argentina</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pereda \textit{et al.}\textsuperscript{40}</td>
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<td>Alvarez \textit{et al.}\textsuperscript{33}</td>
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</tr>
<tr>
<td>Rimondi \textit{et al.}\textsuperscript{41}, Xu \textit{et al.}\textsuperscript{42}</td>
<td>0.25%</td>
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<tr>
<td><strong>Brazil</strong></td>
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</tr>
<tr>
<td>Araújo \textit{et al.}\textsuperscript{39}, Hurtado \textit{et al.}\textsuperscript{35}</td>
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<tr>
<td>Pinto \textit{et al.}\textsuperscript{44}</td>
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<tr>
<td>Hurtado \textit{et al.}\textsuperscript{43}</td>
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<td><strong>Chile</strong></td>
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<td>Mathieu \textit{et al.}\textsuperscript{36}</td>
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<td><strong>Colombia</strong></td>
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<td>Karlsson \textit{et al.}\textsuperscript{38}</td>
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<td>Ghersi \textit{et al.}\textsuperscript{37}</td>
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<tr>
<td><strong>Total</strong></td>
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Table 4. Summary of the number of published AIV strains with each combination of hemagglutinin (HA) and neuraminidase (NA) retrieved from wild birds in South America from 2006 to 2016

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<thead>
<tr>
<th></th>
<th>N1</th>
<th>N2</th>
<th>N3</th>
<th>N4</th>
<th>N5</th>
<th>N6</th>
<th>N7</th>
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<th>N9</th>
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<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>H3</td>
<td>3</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
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<td>1</td>
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<td>8</td>
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<td>2</td>
<td>5</td>
<td>14</td>
<td>4</td>
<td>56</td>
</tr>
</tbody>
</table>

Fig. 4. Distribution of published AIV strains retrieved from wild Anseriformes and Charadriiformes in South America from 2006 to 2016, in relation to the subtypes of hemagglutinin (HA) and neuraminidase (NA). Bar colors represent the continent from where the strains were obtained: South America (black) and North America (grey) (adapted from Krauss et al. 2004).13
Fig. 5. Phylogenetic tree of viral gene sequences, illustrating the five phylogenetic clusters in which influenza A virus strains from mammals and humans are consistently structured (adapted from Hurtado et al.). Trees are drawn to the similar scales, with branch lengths proportional to evolutionary distance.
shifts and reassortment to produce highly pathogenic strains.

Gene sequences are not available for all South American AIV isolates; however, those for which such information is available can be classified in two broad categories: (a) those whose genes are very closely related to those of AIV isolates obtained from wild birds in North America, indicating recent genetic interchange in the Americas; or (b) those presenting one or more gene sequences that are significantly distinct from those found elsewhere in the world, indicating an independent evolutionary pathway. Examples of the first category include AIV isolates obtained from wild birds in northern Brazil, Chile, Colombia and Peru. The second category is mainly represented by AIV isolates obtained from wild birds in northern Argentina, and to a smaller extent northeastern Brazil and Bolivia. An interesting exception is A/seagull/Chile/5775/2009(H13N9), an isolate retrieved from a Franklin’s gull (Larus pipixcan) in central Chile whose gene sequences cluster within the Eurasian clade. A possible explanation is that this strain is closely related to those from other gull species, such as the lesser black-backed gull (Larus fuscus), whose migratory routes extend longitudinally from North America to Europe, hence providing opportunities for intercontinental virus exchange.

More broadly, however, it is fair to state that the genetics of South American isolates corroborate the hypothesis that there are Eurasian and American clades of AIV, and that genetic exchange between these continents is limited. Current evidence indicates there is extensive AIV gene flow between North and South America, which is likely due to the north-south organization of the flyways of migratory birds connecting these continents.

### Implications for poultry health and wildlife conservation

The 2002 avian influenza outbreak in Chile is a clear example of the important role that wild birds can play in the epidemiology of these viruses for the poultry industry. The outbreak of H7N3 in commercial poultry in Chile in 2002 was the first high pathogenicity AIV outbreak reported in birds in South America. The high pathogenicity avian influenza (HPAI) H7N3 strain involved in that outbreak was virtually identical to a low pathogenicity notifiable avian influenza (LPNAI) H7N3 strain that had already been circulating in domestic chickens in Chile, with the exception of an insertion of 30 nucleotides at the HA cleavage site. Because both the HPAI and LPNAI isolates from domestic chicken in Chile were genetically different from AIV isolates from other continents, they were proposed to represent a separate South American lineage, indicating that the virus had been circulating long enough in South America to diverge significantly from the North American or Eurasian viruses. At the time, it was speculated that the original source of the LPNAI strain was wild waterfowl, but unfortunately there were no isolates from local wild birds available for genetic comparison. However, further insight was provided by studies revealing that an LPNAI H7N3 strain obtained from a cinnamon teal sampled in Bolivia in 2001 had gene sequences that were closely related to those of the LPNAI strains from chickens in Chile in 2002. Because other genes of the cinnamon teal strain appeared to be from diverse sources, it was clear that genetic reassortment had occurred. While it was not possible to conclusively demonstrate that the LPNAI strain that was circulating in wild birds had been the original source of the chicken LPNAI strain, this case illustrates that AIV gene flow amongst wild and domestic birds can play a role in the maintenance and emergence of AIV strains that impact the poultry industry.

From a conservation standpoint, there is evidence from studies in other continents demonstrating that in some instances AIV can cause morbidity and mortality of wild birds. This cleavage site of the HA antigen corresponded to that of a low pathogenicity avian influenza (LPAI) strain, although the clinical presentation (acute respiratory distress syndrome, lethargy, oculo-nasal discharge, swelling of the sinuses) and pathological findings (necropsy and histopathological analysis) clearly indicated that the virus was pathogenic for that species. Red-winged tinamous may be unusually susceptible to AIV and therefore their populations could be negatively affected by the spread of the virus, even by AIV strains that would not be theoretically classified as highly pathogenic to avian hosts. Considering the remarkable avian diversity of the South American continent and how little is known with regards to the susceptibility of bird species endemic to the Neotropics, further studies are urgently needed in order to evaluate the susceptibility to AIV infections across orders and families of South American birds (such as Tinamiformes). This concern is furthered by the findings of Ellis et al., who documented the impacts of an HPAI H5N1 outbreak in captive and semi-captive wild birds in Hong Kong, and found that New World species of Anseriformes had a higher influenza-related mortality than those from the Old World.

### Phylogenetic relationship between AIV strains from humans and wild birds

There are no known cases of human influenza in South America that could be directly related to transmission from wild birds. However, it is well established in other continents that wild birds play a key role in the maintenance and transmission of AIV, which may then be transferred to domestic animals and subsequently to humans, and it seems reasonable to suspect that a similar dynamic also takes place in South America. Through the reevaluation of the phylogeny of multiple AIV non-glycoprotein genes from South American AIV strains, it may be noted that isolates retrieved from humans and other mammals in South America can be broadly classified into five main groups (Fig. 5, Table 5; see Hurtado et al.)

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**Table 5. Summary of the phylogenetic clusters of influenza strains retrieved from humans and other mammals in South America (see Fig. 5)**

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Hosts</th>
<th>Countries</th>
<th>Period</th>
<th>Subtypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>equine</td>
<td>Argentina, Brazil, Chile, Uruguay</td>
<td>1963 to 2006</td>
<td>H3N8, H7N7</td>
</tr>
<tr>
<td>B</td>
<td>human, swine</td>
<td>Argentina, Brazil, Colombia, Chile, Ecuador, Peru</td>
<td>2009 to 2014</td>
<td>H1N1, H1N2, H3N2</td>
</tr>
<tr>
<td>C</td>
<td>human</td>
<td>Brazil, Chile, Colombia, Peru, Venezuela</td>
<td>2000 to 2009</td>
<td>H1N1</td>
</tr>
<tr>
<td>D</td>
<td>human</td>
<td>Brazil, Chile, Uruguay, Venezuela</td>
<td>1957 to 1983</td>
<td>H1N1, H2N2, H3N2</td>
</tr>
<tr>
<td>E</td>
<td>human, swine</td>
<td>Argentina, Chile, Colombia, Peru, Uruguay</td>
<td>2007 to 2014</td>
<td>H3N2</td>
</tr>
</tbody>
</table>
a detailed description of the methods).35

Group A comprises H3N8 and H7N7 strains retrieved from horses from 1963 to 2006. The gene sequences of this group are relatively distinct from those identified in avian hosts worldwide, indicating a distinct evolutionary branch. Group A strains are not closely clustered to avian strains, however there are indications that these strains were historically derived from strains of wild birds. The gene sequences of group A are either within the broad North American clade (genes PB2, M and NS) or are in separate evolutionary branches that are shared with South American wild bird strains (genes PA and NP) and/or Antarctic wild bird strains (genes PB1 and PA). These patterns suggest that group A derives from strains that, at some point in the past, circulated and were disseminated by wild birds. An exception in this group is the A/equine/SaoPaulo/4/1976/(H7N7) strain, which presents an unusual sequence for gene M (indicated as A3 in Fig. 5); although this strain’s sequence for the gene M falls within the broad North American AIV clade, for unknown reasons it is highly distinct from all known AIV sequences.

Group B includes H1N1, H1N2 and H3N2 strains retrieved from humans and domestic swine from 2009 to the present. Strains of this group are closely related to AIV strains from turkeys in South and North America, A/turkey/Chile/2831765043/2009/ (H1N1) and A/turkey/Ontario/FAV11417/2009/(H1N1), indicating recent/on-going AIV transmission between mammals and birds. More broadly, the gene sequences of group B strains fall within the North American (genes PB2, PB1 and PA) or Eurasian clades (gene M), indicating past genetic exchange with AIV strains that circulated in wild birds. For genes NP and NS, on the other hand, group B strains are part of separate evolutionary branches that are predominantly composed by strains retrieved from humans in South America but also include strains from chickens and turkeys from North America.

Group C is constituted by H1N1 strains that were retrieved from humans between 2000 to 2009. The gene sequences of the strains in this group are remarkably distinct from those of all known avian strains and consistently are part of separate evolutionary branches, suggesting group C strains are probably specialized to infect humans and not transmitted from/to wild or domestic birds.

Group D comprises H1N1, H2N2 and H3N2 strains that were isolated from humans between 1957 and 1983. The gene sequences of this group are consistently clustered as part of the separate evolutionary branches along with those of group C, with the exception of gene PB1. For this gene, the group is split into three subgroups (indicated as D1, D2 and D3 in Fig. 5) clustering either with strains from domestic birds from Eurasia (D1), with strains of wild birds from Australia and North America (D2), or with strains from group C (D3).

Lastly, group E encompasses H3N2 strains isolated from humans and swine from 2007 to the present. For most genes, the sequences of this group are also part of the separate evolutionary branches along with groups C and D; the only exception was gene PB1, for which group E strains formed a separate evolutionary branch along with group B. Interestingly, two Chinese duck AIV strains frequently clustered along with this group, A/duck/Zhejiang/LS02/2014/(H7N9) and A/duck/Jiangsu/1MA/2008/(H9N2).

When the distinct evolutionary paths of these groups are contemplated in their ensemble, it becomes clear that the gene flow of AIV among birds and humans and other mammals in South America is a fluid mosaic. While some gene segments are part of the North American clade and therefore probably originated from the broader wild bird AIV gene pool that circulates in the Western hemisphere, in most cases these segments are significantly divergent which indicates that they then followed distinct evolutionary paths, with limited genetic interchange with avian strains. The main exception to this broader picture is group B, which comprises AIV strains for which the gene sequences suggest there is still recent/on-going shared transmission among humans, swine and turkeys.

Implications for human health

While strains of the subtypes H1 and H3 are well-established as human pathogens, often with highly efficient human-to-human transmission and relatively low lethality, other subtypes typically associated with birds, such as strains of the subtypes H5 and H7, will tend to be transmitted less effectively among humans but will lead to more severe infections with higher lethality.51,62 This difference in transmission is thought to be related to differences in affinity and anatomical distribution of the sialic acid-linked receptors to which HA binds, with highly pathogenic H5 strains predominantly infecting lower sections of the human respiratory tract.62

Most human cases of H5 and H7 infection have been linked to direct interaction with live poultry, particularly workers of the poultry industry and people visiting live poultry markets.51,63 Less frequent cases of human infections by subtypes H9 and H10, which usually only produce mild disease, have also been linked to contact with domestic birds.54,65 In this sense, it seems probable that the main opportunities for viruses that circulate in wild birds in South America to be transferred to humans would be through domestic animals serving as a bridge for transmission. The phylogenetic evidence for recent/on-going AIV genetic interchange between humans, swine and turkeys (e.g. phylogenetic group B) corroborates this possibility, as does the past evidence for genetic exchange between wild and domestic birds in South America.49

The presence of wild birds entering poultry farms and interacting with domestic birds might therefore play a highly significant role in South America as an interface for genetic flow from wild birds to domestic birds and then to humans. Recent studies have demonstrated that synanthropic species of Passeriformes and Columbiformes may interact directly or indirectly with chickens in poultry farms in South America.66,67 Although no evidence of AIV infection was detected thus far in such synanthropic birds near poultry facilities, such studies stress the need for the development and implementation of biosecurity measures that effectively prevent wild birds from closely interacting with poultry in farms.69

Although domestic birds may pose the most significant risk in terms of AIV gene flow from wild birds to humans, the possibility of direct AIV transmission from wild birds to humans should not be dismissed. For instance, traditional communities from some regions of South America may engage in cultural practices that provide opportunities for the transmission of AIV. One such example is the illegal trade of game meat in some regions of the Amazon.68 Another example are the remote fishing communities along the Amazon coast of Brazil, where capturing and maintaining aquatic birds, captive in precarious domestic environments, is a common practice, whether for meat consumption (Anseriformes) or to serve as pets (Charadriiformes).69 It is acutely concerning that in one community where this practice is frequent, the swab of a captive white-faced whistling duck (Dendrocygna viduata) had detectable traces of AIV genetic material.43

Besides indigenous and traditional communities that hunt or interact otherwise with wild birds throughout the continent, sport hunters may also be at risk. For instance, Alvarez et al. identified AIV as the cause of death of red-winged tinamous at hunting grounds.33 This occurred less than 50 km from Buenos Aires
strategic component in preparing for and responding to influenza is, therefore, clear that AIV surveillance in wild birds should be a strategic component in preparing for and responding to influenza epidemics in humans in South America. Lastly, it is worth noting that researchers, rehabilitators, veterinarians, conservationists and other professionals working with wild birds may also be at risk of exposure, and should at all times adopt biosecurity measures while working with these animals, especially when it comes to the use personal protective equipment.

**Directions for the future**

The fragmented and discontinuous nature of research efforts and the lack of long-term national and international surveillance programs have limited our understanding of the regional characteristics of AIV ecology in South America. The South American continent has unique ecosystems, avian communities and environmental conditions, which may potentially lead to differences in the ecology and epidemiology of AIV in relation to other well-studied regions, such as North America, and, therefore, we cannot assume that data from other continents can be extrapolated to South America without the need of confirmatory studies.

For these reasons, while it is vital to conduct targeted surveillance of bird groups that are well-established as reservoirs of infection for these viruses, namely Anseriformes and Charadriiformes, moving forward it will also be important to examine whether other avian taxa could play a distinct role in the ecology of AIV in South America. In particular, groups such as Phoenicopteriformes (flamingoes), Procellariiformes (albatrosses, petrels), Sphenisciformes (penguins) and Suliformes (cormorants, gannets) have characteristics and behaviors that could lead them to play a role in the epidemiology of AIV. Furthermore, it is also worth examining whether South American endemic groups of terrestrial birds (e.g. Tinamiformes, Rheiformes) are susceptible to AIV infections.

With regards to the geographic distribution of the study effort, surveillance will benefit from monitoring stop-over and wintering sites along the flyways of migratory aquatic birds. However, considering the ecological and landscape diversity of the continent, it is important to also dedicate sampling effort to other areas to evaluate regional and biome-specific differences in AIV epidemiology. Additionally, valuable insight may be obtained by investigating the occurrence of AIV in wild birds in areas where there might be a closer interaction among humans, domestic and wild birds, such as swine and poultry farms, hunting grounds, and communities where subsistence hunting and domestication of wild birds is common practice.

Lastly, it is important to underline that the failure of many publications to provide detailed information on the sampling effort has limited a comprehensive meta-analysis of the literature on AIV in wild birds in South America. Although it is understandable that scientific journals need to impose restrictions on article length, authors should bear in mind that supplementary files are a valuable alternative to provide detailed datasets that greatly extends the value and contribution of any given publication. We, therefore, urge researchers to use supplementary files to provide extensive details on the sampling effort (e.g. species, location, GPS coordinates, date, etc. for each sample) and diagnostic results of their studies (e.g. cycle threshold values, detailed pathobiology testing results, etc.). Such details should be provided not only for samples with confirmed positive results but also for samples that were positive or inconclusive in screening tests (e.g. rRT-PCR) but that could not be confirmed by subsequent tests, and also for samples that were negative. Furthermore, it is essential to make every effort to sequence all eight genome segments of the virus of every AIV isolate, and to deposit these sequences in publicly-available databases (such as GenBank).

**Conclusion**

Although avian influenza virus is one of the most studied pathogens in the world, studies on the ecology and epidemiology of these viruses in South America are few and fragmented. Current evidence corroborates that many of the broad epidemiological patterns that have been documented in other continents, such as the role played by Anseriformes and Charadriiformes in the maintenance and spread of AIV, are also true in South America. On the other hand, the fact that AIV prevalence in South American studies appears to be remarkably lower than that observed in other continents, along with the presence of endemic taxa of birds that may be highly susceptible to AIV, indicates that South America may have distinct characteristics that modulate the epidemiology of AIV in unique ways. The last decade has witnessed the emergence of a South American research community dedicated to investigating this virus, resulting in a significant number of studies being published in recent years. However, our knowledge on the occurrence of avian influenza in South America is still limited and there are important gaps in the species and geographic distribution of the sampling effort.

**Acknowledgments**

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**Conflict of interest**

The authors have no conflict of interests related to this publication.

**Author contributions**

Compiled and analysed the data (RH, RETV), drafted and revised the manuscript (RH, RETV).

**Supplementary information**

(Supplementary information is linked to the online version of the paper on the Exploratory Research and Hypothesis in Medicine website.)

**References**


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“Hello Father…Hello Mother…Hello…Mother?”
3-Person Cytoplasmic Transfer: Mitochondrial Genomes Redefine In Vitro Fertilization (IVF) for a Healthier Family in the Twenty-First Century

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1063 Mosser Rd., B194 Breinigsville, PA 18031, United States

Abstract
In 1978, the idea of making a baby on a petri dish generated worldwide media attention as scientific and social controversy. On July 25th of that year, the world’s first human in vitro fertilization (IVF) was accomplished in England, and Louise Brown was the first ‘test-tube’ baby. Once Louise was shown to be a healthy infant the protests subsided, and since then the IVF technique has gradually become accepted as an alternative to the ‘natural’ way for infertile couples to have a child. However, IVF is not without risks and possible disappointment, and for 37 years there has been no significant change in technique. However, in 2006 two colleagues in England developed a novel method for IVF that will eliminate inherited disease by using a woman’s donated, healthy mitochondrial (mt) organelles (oxygen-energy cells), euginizing the mother’s unhealthy mt cells that carry inherited chronic and/or serious disease. The transfer of these powerful, healthy cells into the mother’s egg cell represents a 3-person IVF process by which many chronic and serious diseases are eliminated that would otherwise have been passed on by the mother to her child. In cytoplasmic (mt) implantation, the mother’s cell nucleus is not affected and any characteristics of the child, for example hair and eye color, are from the mother and father. This technique has been successfully researched in mice, but to date not in humans. This year, the United Kingdom petitioned Parliament and was granted approval to research on humans. In the United States, the Food and Drug Association (FDA) has been reluctant to give human research approval for infertile couples to have a child. Media and the public were uncomfortable against “laboratory breeding, unknown long-term health risks, socially ostracizing a child, and destruction of the nuclear family”, to name only a few of many concerns vehemently voiced.1-7 My colleague’s off-hand reply was not meant to be sarcastic or to suggest an inappropriate tryst, but the idea of three IVF parents does at first sound bizarre. Yet, this is far from the case.

In vitro means ‘outside the body’. Fertilization means sperm has attached to and entered the egg. Fertilization of an egg outside the body first began by experimenting with rabbits in 1932. By 1944, human ova were successfully fertilized in a petri dish but not implanted in a woman. The birth of the first ‘test tube’ IVF baby, Louise Brown, occurred on July 25, 1978 in England, accomplished after 4 years of unsuccessful challenges for her parents, scientist Robert Edwards, and Patrick Steptoe, a gynecologist and surgeon.1,2,3,8 At the time, ethical questions flourished, cautioning against “laboratory breeding, unknown long-term health risks, socially ostracizing a child, and destruction of the nuclear family”, to name only a few of many concerns vehemently voiced.1,2,3,5-10 Indeed, to this day, the Catholic Church remains opposed to IVF.

In 1977, England transformed IVF and possibilities for future couples to have a child. Media and the public were uncomfortable and skeptical with the idea of creating a child not by sexual procreation, but instead by technical science, raising valid concerns then and currently. Some that persist include: Would this process lead to conceiving children as “quality controlled products”? What if the child was handicapped mentally or physically? What about long-term health?2,3,7,9

‘Mighty-mouse’ mitochondrial organelles: what are they?

Advances in early 21st century research led to the discovery of the powerful ability of mitochondrial enzymes to block maternal transference of many inherited illnesses and diseases. Mitochondria are tiny organelles within each cell of the body, regulating 79% of a

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cell’s oxygen in order to sustain growth.\textsuperscript{3,7} By about 2006, scientific research had discovered the significant influence of these tiny organelles, thousands within each cell, and their ability to block many inherited diseases and chronic illnesses. The research also determined that not all mitochondria are healthy and, when not, they can cause inherited chronic illness or disease in children and later, even in adulthood.

What is inherited mitochondrial disease?

The Foundation of Mitochondrial Medicine states on their website (http://mitochondrialdiseases.org/related-diseases/): “Mitochondrial disease can look like any number of better known diseases, including: autism, Parkinson’s disease, Alzheimer’s disease, Lou Gehrig’s disease (ALS), muscular dystrophy and chronic fatigue syndrome, among others. Adults and children with it can have features similar to other disorders like: epilepsy, myopathy, developmental delay, learning disabilities and fibromyalgia.”\textsuperscript{7}

In pediatrics and adult medicine, unhealthy, low-oxygenated mitochondria and mtDNA can cause cardiac, respiratory, gastroenterological (GI), endocrinal and parathyroid diseases, as well as poor growth, decreased muscle coordination, seizures, and cognitive and physical development delays, to name only a few of the potential pathological manifestations.\textsuperscript{3,6,11} Teratology related to unhealthy mitochondria organelle low-oxygen levels affect an evolving cell and can make a difference between a healthy child or their inheriting chronic illness or a serious disease, having lifelong consequences for the child and the family caregivers.

Mitochondrial disease cannot be cured, and mutated mtDNA results in 25%–30% of mitochondrial disease.\textsuperscript{7,12} It is estimated that 1 in 2,000 children in the United States will be born with or eventually develop an inherited disease that is caused by unhealthy mitochondria passed on by the mother. Cytoplasmic replacement, or replacing unhealthy mitochondria in the mother’s egg with the healthy mitochondria of a female donor, effectively ‘eugenizes’ the unhealthy mitochondria and eliminates whatever inherited illness only the mother would pass on.\textsuperscript{1,2} It is important to note that the third person’s donated mtDNA is not responsible for the characteristics of the child; for example, the child’s hair and eye color and bodily appearance are their biological parent’s characteristics because the mother’s nucleus is not touched by the transfer of unhealthy mitochondria with the donor’s healthy organelles.\textsuperscript{1,4}

What is the chance that other siblings will be affected if the mitochondria are not replaced?

Autosomal inheritance involves the 22 pairs of chromosomes not concerned with determining the sex of a child. If a gene trait is recessive (one gene from each parent), often no other family members will appear to be affected; although, there is a 25% chance of the trait occurring in other siblings. If a gene trait is dominant (one gene from either parent), often the disease will occur in other family members; there is a 50% chance of the trait occurring in other siblings, the symptoms being either more or less severe, or the disease not developing until later in life, such as with type 2 diabetes or Parkinson’s disease. Moreover, some siblings are more afflicted than others. The possibility of mitochondrial toxins can also cause acquired warning signs, and overall the prognosis of mitochondrial diseases is unpredictable and, as stated above, there is no cure.\textsuperscript{3,6,11,12}

How are mitochondrial chronic illnesses and/or diseases diagnosed?

Diagnostic assessment is done by a system that is represented by one (or more) of the following three categories:\textsuperscript{6,12}

1. **Metabolic testing:** Urine profiles, blood protein oxidative phosphorylation (OXPHOS), and adenosine triphosphate (ATP) processing. Through these molecules and processes, mitochondria turn the food we eat into energy to be utilized by major organs. OXPHOS, however, is not currently considered as adequate for diagnosis on its own.
2. **Muscle and liver tissue pathology.**
3. **Genetic testing:** mtDNA, nuclear DNA.

Criteria categorization of diagnosis

**Possible**

For the three tests described above, one is abnormal and the other two are normal or equivocal.

**Highly probable**

Greater than two tests are abnormal.\textsuperscript{6,12} It is important that the patient be evaluated by a physician who is experienced in mitochondrial diseases or syndromes, particularly if there is a recognizable clinical syndrome. See web list of mitochondrial medicine specialists across the country.\textsuperscript{12}

What are the treatments for mitochondrial disease?

A ketogenic diet is suggested for patients with lower severity disease. Arginine, a supplement which increases nitric oxide production and results in vasodilation during resistance exercise therapy, has not been as effective as was hoped. Symptom management is appropriate for problems as they arise, such as seizures, diabetes mellitus and cardiac conditions, but the mitochondrial disease itself is unchanged.\textsuperscript{6,12} The newly developed EPI-743 is a medication that could benefit children with a variety of mitochondrial diseases, and the manufacturer Edison Pharmaceuticals has entered into a partnership with Dainippon Sumitomo Pharma Co., Ltd of Japan to join in research for pediatric mitochondrial diseases, beginning with EPI-743.

How is donor replacement accomplished in 3-person IVF?

Mitochondrial donor replacement goes by several names: nuclear genome transfer, cytoplasmic transfer, and genetic modification of mitochondria organelles.\textsuperscript{10} Replacing the unhealthy mitochondria with healthy mitochondria of a donor is effective to exclude the possibility of a mother passing on inherited disease or chronic illness related to the organelle’s genome. The tiny mtDNA organelles, thousands in each cell, are the ‘powerhouse’, or the batteries, that send proteins and energy to the nucleus of each cell; by replacing the mother’s unhealthy mitochondrial enzyme with a 3rd person female’s healthy mitochondrial enzyme, the inherited disease genes are eliminated from the process.\textsuperscript{1,3,6,11,13}

There are five stages of 3-person IVF. If at stage 4, multiple
embryos have developed, a single cell is removed from each and screened for genetic disorders, allowing the parents to implant only those embryos free of genetically-carried disease.1,3,6,8,10,14,15

Probable risks: physically, emotionally and ethically

Research in embryonic extension anticipates preventing birth defects and advancing prenatal care. One question of concern is: are there higher rates of disease in IVF children? Although, in 2016, IVF has matured to a point that it is now considered an accepted and common alternative to a couple’s inability to conceive ‘naturally’, emotional and physical preparation is paramount for the parents and for a mitochondria donor in a case where the IVF would be a 3-person procedure. IVF may not be successful in either a 2-person or potential 3-person technique.1,3,8,9,12

In the United States, the FDA has not yet approved mitochondria cytoplasmic transfer, and the regulatory agency is holding off until more is known about the safety. A non-profit enterprise called Mothers for a Human Future cautions “awareness, advocacy, and activism about human biotechnologies that could alter the human species”. Founder and Director, Enola G. Airds, in a letter dated 10/17/2013, urged the FDA to reject the request to allow clinical trials on germine mitochondrial techniques. Among the objections she lists are questions of ethical, social and legal concerns. Additionally, there is a concern that ‘designer babies’ could be the goal of some potential parents.10,16,17 James Watson, co-discoverer of the DNA structure, had expressed to pioneer colleague Robert Edward’s that he is not completely against IVF but “infanticide could occur”; coordinately, he asked, “…and what are we going to do with the mistakes”?2

Paramount to the parents investigating IVF is informed consent and verbal understanding of the drawbacks. Physical health side-effects, emotional disappointment and ethical considerations are major counseling issues, including their legal implications.1,9,10,16-21 With 3-person IVF on the forefront, these issues become more complicated.9,17,21 For some, the desire to have a family is so overwhelming that the risks of IVF to health and partnership are often met with selective hearing or even discounting that the embryos could ever be a source of division between the couple themselves or the donor. Several states have policies in place to protect the IVF child and parents, but these are based on the 2-person IVF and a clinic’s policies. All states do not share common legal policies, and such is the case in foreign countries.

Common health risks of IVF include bleeding, infection, damage to bowel or bladder, possible multiple pregnancies, premature delivery, low birth weight and unsuccessful development of viable, even in cases of successful IVF. An additional significant concern is the great expense of IVF, currently at $12,000–$17,000 per one cycle in the United States. Success also depends on a diverse array of factors, including reproductive history, maternal age, cause of infertility and lifestyle factors. And, ultimately, pregnancy rates from IVF are not the same as live birth rates.18,19

Parentage and embryo outlook: legal issues

In the case of multiple embryos, a key question is: what do we do with the unused embryos?22,18 There is the option of embryo banks using cryopreservation (freezing) until a carrier is found through charitable organizations. A childless couple can legally adopt the embryos for their own IVF procedure (this is another means of 3rd party reproduction). The couple or, in today’s society, the single person who chooses to give up the additional, unused embryos releases all rights to them or any child issuing from the IVF and will have no knowledge of the new parents.18,19 There have already been several legal battles between partners to have or refuse to give custody of unused embryos, and 3-person IVF will further engage legal parameters.

Summary

The adage of “no risk, no gain” may apply to IVF. Indeed, science itself would not have advanced if risks had not been taken. Medical “miracles” like penicillin and vaccines, such as those to prevent polio, bacterial meningitis, measles, pertussis, chickenpox, shingles, pneumonia, tetanus and hepatitis, would not have been developed without the pioneering scientists and research efforts that included challenge in monitored trials on humans. IVF was once a medical and societal storm, until Louise Brown’s creation in a “test tube” led to a healthy birth and, so far, a healthy life. It is estimated that in the United States 2,000 births a year are the result of IVF, making a family unit possible for childless partners.

As we look at fertility options since Louise Brown’s birth, it has taken 37 years for any significant advancement. Healthy mitochondrial cells transferred during IVF can be the future of healthy generations, eliminating many inherited chronic conditions and diseases.1,2 In the United States, the controversy surrounding the pros and cons of this, however, continues to debate in scientific and societal conversations.

Question

If, in the next few decades, research scientists could ensure the purging of many inherited chronic and serious diseases through healthy donor mtDNA transfer, can we afford to not proceed?

Conflict of interest

The author has no conflict of interests related to this publication.

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