Peer Review File
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Reviewer A

This is a well-presented review of the current status of NGS in Rh blood group genotyping. The paper provides us with nearly complete information on the progress of research in NGS strategies used for RH genotyping. As a review, it summarizes current studies of this application and points towards future directions. My comments on the manuscript which is already of a high standard are quite minor.

Minor points of improvement:

1. Please, use correctly names concerning antigens, blood groups/systems, phenotyping (without italics) (e.g. Rh) and alleles, genes, genotyping (italics, capital letters) (e.g. RH).

Response : thank you for this correction, we agree and have made the necessary revisions.

Changes in the text : multiple corrections throughout the manuscript, marked in the 'tracked changes' version.

2. The section "Abstract" line 42 and 43: "WGS" and "ES" are not explained correctly. In my opinion, there is no need to use the abbreviations here. There are explained in the main text.

Response : The abreviations have been removed and the text has been modified for clarity.

Changes in the text : line 43

3. Line 150-152 It is not clear where the data come from (I suspect Table 2?) - please clarify.

Response : Line 153 now indicates Table 2 as the source of the information for the paragraph.

Change in the Text : The reference (Table 2) was moved to the previous sentence on line 153.

4. Line 152: 0.25% - is it a correct calculation?

Response : The error has been corrected. The inclusion of four more studies modifiy some of the percentages previously calculated. Values have been changed when necessary to account for the inclusions.

Changes in the text : throughout the paragraph within lines 151 to 159.

5. The section " Social, ethical, and legal considerations" is very important but the content is too long (3 pages) since it is general for NGS of all blood group systems not

limited to Rh system. The section will benefit when the text is more concise.
Response : We agree with the reviewer and have reduced the length for this section of the manuscript.
Chages in the text : Throughout the specific section.

6. To the lines 278-283: The RH NIPT using NGS was described in ref no. 55 and the publications: Takahashi K, et al. Clin Chem 2019;65:1307-16; Wienzek-Lischka S et al. Transfusion 2015;55:1538-44; Wienzek-Lischka S, Transfus Med Hemother 2020;47:14-22; Rieneck K, et al. Prenat Diagn. 2021;411380-1388 More precise state of RH NIPT should be added.
Response : The bibliography has been added and commented in the manuscript.
Changes in the text : The new reference numbers are 67 to 70.

7. The manuscript will benefit when some more publications are mentioned and discussed such as: El Wafi M, et al. Novel intronic RHD variants identified in serologically D-negative blood donors. Vox Sang 2017;112:796-802; Dinardo CL, et al. Diversity of RH and transfusion support in Brazilian sickle cell disease patients with unexplained Rh antibodies. Transfusion 2019;59:3228-35.
Response : The publications have been added to Table 2 and to the manuscript text.
Changes in the text : Table 2 and multiple instances throughout the manuscript.

8. Add the reference numbers in Table 2.
Response : Column 1 of Table 2 includes now the reference number.
Changes in the text : Column 1, Table 2

Reviewer B

This review covering RH genotyping by Next Generation Sequencing provides a comprehensive account of the state of the art on this topic. It is very well structured, and covers in depth the literature across scientific, technical, clinical as well as social, ethical and legal domains. This review should serve as an excellent reference point as the topic expands in the future. It was a pleasure to review such as scholarly, well researched and well-presented manuscript. There are only a few comments:

1. Under Copy number determination: Line 114: Please provide a reference to support the statement about the duplication exon 3 being ‘detected through NGS copy number analysis.
Response: We have added the reference for Stef et al, 2020. This manuscript’s NGS methodology was validated for the detection of RHD*01W.150 (duplication of RHD exon 3) as listed in the cited manuscript Supplement S8: sample set. In addition, our own unpublished clinical experience has demonstrated the detection of this allele by NGS but not by targeted SNV-based probe hybridization assays.
Changes in the text: reference number 37 added to line 115.

2. Under Social, ethical, and legal considerations: Line 347: with regard to the paragraph with the sentence commencing: "Although the RH system is not sex-linked, participants being tested are often asked about their sex…." Can this sentence be referenced? The meaning of this first sentence and the link between RH and the next sentence is not clear. Could the authors clarify the ideas being expressed in this paragraph. Minor

Response: This paragraph was removed from the manuscript as RH is not X-chromosome coded.

Changes in the text: Paragraph removed.

3. Under Social, ethical, and legal considerations – Line 293- suggest spell out 'GENCOV'

Response: This acronym has been removed as a consequence of shortening the section, as requested by the previous reviewer.

Changes in the text: the original text corresponding to this acronym has been removed.

Reviewer C

The authors present a very useful review of RH genotyping by next generation sequencing, considering not only the scientific aspects but also the social, ethical and legal aspects. Table 2, in particular, highlights the depth of work that has been completed to date in this area and allows the reader to link to various resources that are needed for this type of approach.

Minor comments:

Gene names should be in italics throughout text, figures, figure legends and tables – there are many places where this is not the case.

Response : thank you for this correction, we agree and have made the necessary revisions.

Changes in the text : multiple corrections throughout the manuscript, marked in the 'tracked changes' version.

Line 152 – ES publications should be 25% (6 of 24) and not 0.25%.

Response : The error has been corrected. The inclusion of four more studies modifiy some of the percentages previously calculated. Values have been changed when necessary to account for the inclusions.

Changes in the text : throughout the paragraph within lines 151 to 159.

Line 154 – the long range PCR approaches are also PCR-amplification and so it would be better to state 6 studies are PCR amplification.

Response: we added additional studies and consolidated the 'PCR' and 'long-range PCR' categories as a single 'PCR amplification'.

Change in the text: line 155

Lines 157/158 – potentially link the custom targeting approach with the issue of similarity between the RHD and RHCE sequences and comment on the problem this causes.
Response: the complex and detailed advantages and disadvantages of each custom targeted approach in relation to paralogous gene misalignment are unfortunately beyond the scope of this section. For example, pre-amplification with gene-specific primers with subsequent masking of the paralogous gene may resolve mapping accuracy. On the other hand, probe-capture to paralogous regions or non-specific exon amplification require bioinformatic approaches to resolve this. Throughout the manuscript, we refer to specific publications that demonstrate each of these different approaches and also discuss the corresponding data analysis approaches.
Changes in the text: none

Line 213 – should it be 66% 'accuracy'? Please comment on why Rh- accuracy is lower.
Response: we cite the accuracy values reported verbatim in the published manuscript. A discussion of the limitations of the BOOGIE algorithm is beyond the scope of this review, but we do provide readers with a comprehensive list and description of subsequent bioinformatic tools with reported improved accuracy in the *RH* locus.
Changes in the text: none

Line 228 – include in text citations after authors et al and not at end of sentence on line 230.
Response: this change has been addressed.
Change in the text: lines 231, 232, and 236.

Lines 244-247 – not all of the listed information is included and cross-referenced to Table 2; potentially include another column in Table 2 to highlight the areas discussed here.
Response: Publication references are provided for each of the items listed. Additional columns for Table 2 were considered, but not adopted to maintain readability and simplicity of the material.
Changes in the text: none.

Line 280 – missing Finnish study:
Haimila, K, Sulin K, Kuosmanen M, et al. Targeted antenatal anti-D prophylaxis 402 program for RhD-negative pregnant women – outcome of the first two years of a 403 national program in Finland. Acta Obstetricia et Gynecologica 404 Scandinavica. 2017;96(10), 1228-1233.
Response: this citation has been included (reference #63).
Change in the text: line 291.

Line 285 – please consider rephrasing subheading as this section appears to be more general and not specific to RH typing.
Response: We have rephrased the title to reflect that this section addresses social, ethical and legal considerations on the use of NGS for blood typing in general.
Change in the text: subsection title edited to 'Social, ethical, and legal considerations for NGS in blood typing'.

Table 1 – studies cited need reference numbers with full details included in reference list.
Response: we have added the reference numbers to column 1.
Change in the text: Table 1, column 1 now includes reference numbers.

Table 2 – as above for Table 1.
Response: the citations have been included.
Change in the text: citation number has been added in column 1, Table 2.

Table 2, Giollo et al., 2015 – check formatting at ends of row.
Response: formatting has been reviewed
Change in text: NA

Table 2 – various typos need correcting:
Fichou et al., 2014 – introns
Schoeman et al., 2018 – unresolved
Lane et al., 2108 – prediction
Chang et al., 2020 – profiling
Response: thank you for the observations, these have been all corrected.
Changes in text: as indicated in the reviewer comments (Table 2)

Why is Giollo et al., 2015 'presumably RHCE included as well'?
Response: the actual results description in the manuscript (Giollo et al 2015) only refer sto 'Rh+' and 'Rh-', and discuss hybrid *RHD* alleles where *RHCE* exons are translocated into the *RHD* gene. This information does not confirm that *RHCE* variants were interpreted as well. Figure 3, which lists all the interrogated blood groups, lists 'RH'. The introduction does describe the C/c/E/e antigens appropriately, so we presumed those were included in the analysis and and in the overall concordance and coverage reported results; however no specific data is included in the results for *RHCE* alleles or antigens to confirm this was the case.
Changes in text: none

Table 2 – it would be useful to have a column stating whether novel alleles/variants had been discovered in a study – to link with line 104.
Response: Thank you for this input. Indeed, a number of additional columns for Table 2 were actively considered, but not adopted to maintain readability and simplicity. We

have added to this line the exact list of references that reported novel alleles to direct the reader to the detailed information.
Changes in the text: added references to lines 105-106.

Table 2 is missing the following study:
https://pubmed.ncbi.nlm.nih.gov/35652461/
Response: this citation has been included.
Change in the text: reference 36.

Line 426 – 1st author is 1000 Genomes Project Consortium
Line 447 – check author listing
Line 480 – check formatting of citation
Line 526 – details missing
Line 621 – details missing
Response: all these comments have been addressed
Change in the text: as indicated by the reviewer comments

Reviewer D

General comments:
This review discusses challenges and opportunities for RH sequencing with next-generation methods. Sequencing of the RH loci has always been a challenge. Next-generation sequencing technologies have features that may facilitate novel approaches in RH sequencing. Therefore this review is of interest to the scientific community.

It might be helpful for the reader if the authors would include a brief description and possibly also an image about the RH genomic organization.
Response: we appreciate this suggestion, however the description and schematic figures of the RH locus are available in multiple sources of the literature, and we refer the reader to these citations early in the manuscript. We propose to focus our manuscript to figures and Tables that contribute new content to the literature. In addition, since this review manuscript will be published as a part of a series that focused on the molecular study of the RH locus, such a figure would likely be repetitive from accompanying papers.
Change in the text: none

We have found it very difficult even with long-read techniques to unambiguously sequence the RHD locus due to the size and homology with RHCE. I would recommend to describe the technical challenges and opportunities more concisely as these are in my view still substantial.
Response: we have expanded the 'NGS chemistries and library preparation' section to include a broader discussion of long-read sequencing applications, and cited recent published references that review this topic in depth (Thun et al, 2023, reference 40)

Change in the text: lines 160-166 and reference 40

I would also take the costs of NGS sequencing into consideration. Still library preparation and sequencing can be rather expensive if the assays are not thoroughly optimized for throughput. Also the turn-around times are usually long. In which way does this impact point of care diagnostics?

Response: NGS costs have been steadily decreasing, and new technologies with very low cost have been announced in the market for the near future. Therefore, we describe this trend in the manuscript and provide the reader with a reference for published costs over time (NHGRI, 'the cost of sequencing a human genome', reference 3). As the reviewer mentions, cost per test is related to throughput, but since these values are not static such a discussion is beyond the scope of this review. For example, a new platform that recently emerged in the market (sequencing by avidity) advertises a low cost that is attainable without the need for high-throughput testing, unlike other short-read sequencing-by-synthesis NGS platforms.

Change in the text: line 64, reference 3.

Another serious problem is the occurrence of PCR artifacts in homologous genes if PCR based methods are used. I would also mention this as a limitation.

Response: use of PCR is mentioned as one of the approaches for targeted NGS, and the cited manuscripts include approaches that range from gene-specific amplification, to non-specific amplification with bioinformatic approaches to resolve paralogous gene assignment, to the use of read depth and split reads to resolve complex rearrangements. We believe this addresses the complex advantages and disadvantages of the PCR approach.

Change in the text: none