RESEARCH ARTICLE

Novel RNA viruses associated with *Plasmodium vivax* in human malaria and *Leucocytozoon* parasites in avian disease

Justine Charon1, Matthew J. Grigg2,3, John-Sebastian Eden1,4, Kim A. Piera2, Hafsa Rana4, Timothy William3,5,6, Karrie Rose7, Miles P. Davenport6, Nicholas M. Anstey2,3, Edward C. Holmes1*

1 Marie Bashir Institute for Infectious Diseases and Biosecurity, Charles Perkins Centre, School of Life and Environmental Sciences and Sydney Medical School, The University of Sydney, Sydney, New South Wales, Australia, 2 Global and Tropical Health Division, Menzies School of Health Research and Charles Darwin University, Darwin, Northern Territory, Australia, 3 Infectious Disease Society Kota Kinabalu Sabah – Menzies School of Health Research Clinical Research Unit, Kota Kinabalu, Sabah, Malaysia, 4 Centre for Virus Research, Westmead Institute for Medical Research, Westmead, New South Wales, Australia, 5 Clinical Research Centre – Queen Elizabeth Hospital, Kota Kinabalu, Sabah, Malaysia, 6 Gleneagles Hospital, Kota Kinabalu, Sabah, Malaysia, 7 Australian Registry of Wildlife Health, Taronga Conservation Society Australia, Mosman, New South Wales, Australia, 8 Kirby Institute for Infection and Immunity, University of New South Wales, Sydney, New South Wales, Australia

* edward.holmes@sydney.edu.au

Abstract

Eukaryotes of the genus *Plasmodium* cause malaria, a parasitic disease responsible for substantial morbidity and mortality in humans. Yet, the nature and abundance of any viruses carried by these divergent eukaryotic parasites is unknown. We investigated the *Plasmodium* virome by performing a meta-transcriptomic analysis of blood samples taken from patients suffering from malaria and infected with *P. vivax*, *P. falciparum* or *P. knowlesi*. This resulted in the identification of a namavirus-like sequence, encoding an RNA polymerase and restricted to *P. vivax* samples, as well as an associated viral segment of unknown function. These data, confirmed by PCR, are indicative of a novel RNA virus that we term Matryoshka RNA virus 1 (MaRNAV-1) to reflect its analogy to a “Russian doll”: a virus, infecting a parasite, infecting an animal. Additional screening revealed that MaRNAV-1 was abundant in geographically diverse *P. vivax* derived from humans and mosquitoes, strongly supporting its association with this parasite, and not in any of the other *Plasmodium* samples analyzed here nor *Anopheles* mosquitoes in the absence of *Plasmodium*. Notably, related bi-segmented namavirus-like sequences (MaRNAV-2) were retrieved from Australian birds infected with a *Leucocytozoon*—a genus of eukaryotic parasites that group with *Plasmodium* in the Apicomplexa subclass hematozoa. Together, these data support the establishment of two new phylogenetically divergent and genomically distinct viral species associated with protists, including the first virus likely infecting *Plasmodium* parasites. As well as broadening our understanding of the diversity and evolutionary history of the eukaryotic virosphere, the restriction to *P. vivax* may be of importance in understanding *P. vivax*-specific biology in humans and mosquitoes, and how viral co-infection might alter host responses at each stage of the *P. vivax* life-cycle.
Author summary

While parasites are a major cause of human disease, they can themselves be infected by viruses. We asked whether three of the major malaria-causing parasites in humans—Plasmodium vivax, P. falciparum and P. knowlesi—were also infected by viruses. To this end we performed total RNA-Sequencing (“meta-transcriptomics”) on human blood samples infected with these Plasmodium species. This resulted in the discovery of an abundant bi-segmented virus—Matryoshka RNA virus 1 (MaRNAV-1)—in all P. vivax samples tested (but no other Plasmodium species) that contains a replicase segment related to those of narnaviruses, arguably the simplest type of RNA viruses discovered to date. By screening for MaRNAV-1 in a larger set of Plasmodium species we revealed a strong specificity between this virus and P. vivax, as well as the presence of a related virus—MaRNAV-2—in avian Leucocytozoon hematozoa parasites. This is the first discovery of a Plasmodium-associated virus and will assist in revealing the deep evolutionary history of RNA viruses and our understanding of Plasmodium biology and disease processes.

Introduction

Viruses are the most abundant biological entities on Earth, replicating in diverse host organisms [1]. Although there has been an expansion of metagenomic studies dedicated to exploring this immense virosphere [2–6], our knowledge of the viral universe remains limited, with only a minute fraction of eukaryotic species sampled to date [7]. This knowledge gap is especially wide in the case of unicellular eukaryotes (i.e. protists), including those responsible for parasitic disease in humans, on which only a small number of studies have been performed.

Viral-like particles in parasites were first observed by electron microscopy as early as the 1960’s in various protozoa from the apicomplexan and kinetoplastid phyla [8]. The first molecular evidence for the existence of protozoan viruses was obtained in the late 1980s, resulting in the characterization of double-strand (ds) RNA viruses in the human parasites Giardia, Leishmania, Trichomonas and Cryptosporidium [9–14]. More recently, single-stranded narnavirus-like and bunyavirus-like RNA viruses were identified in trypanosomatid parasites, including Leptomonas seymouri, Leptomonas moramango, Leptomonas pyrrophocoris and Crithidia sp. [15–18]. However, our knowledge of protozoan viruses is clearly limited, with many of those identified stemming from fortuitous discovery.

The identification and study of protozoan viruses is also important for our understanding of so-called “Russian doll” (“Matryoshka” in Russian) infections [19], in which parasites are themselves infected by other microbes. A key question is whether viruses of parasites can in turn have an impact on aspects of parasite pathogenesis? An increasing number of studies have demonstrated that dsRNA viruses of protozoa can affect key aspects of parasite biology, including their virulence, in a variety of ways [20]. For instance, data from Leishmania guyanensis and Trichomonas vaginalis strongly suggest a link between parasite pathogenesis and the presence of Leishmania RNA virus 1 (LRV1) and Trichomonas vaginalis virus, respectively [21–23]. By increasing the inflammatory response in the host these viruses could in theory enhance human pathogenesis [24–25]. Interestingly, associations have also been observed between LRV1-infected L. guyanensis or L. braziliensis and treatment failure in patients with leishmaniasis [26–27].

Viral co-infection also has the potential to alter protozoan biology and/or attenuate the mammalian host response, leading to greater replication or persistent protozoan infection, in turn promoting ongoing parasite transmission. Persistence (i.e. avirulent infection) has been
proposed in the case of Cryptosporidium parvum virus 1 (CSpV1) that infects the apicomplexan Cryptosporidium [28], and increased C. parvum fecundity has been demonstrated in isolates experiencing viral co-infection [29]. Viral infections may also have a deleterious effect on parasite biology, adversely impacting such traits as growth and adhesion in the case of axenic cultures of Giardia lamblia [10]. Clearly, the effects and underlying molecular basis of any consequences that protozoal viruses have on their hosts, including in the context of pathogenesis, requires rigorous investigation. Documenting novel protozoal viruses is an obvious first step in this process.

Remarkably, nothing is known about those viruses that infect species of Plasmodium (order Haemosporida)—obligate apicomplexan parasites of vertebrates and insects. In vertebrate hosts, these protozoa first infect the liver cells as sporozoites where they mature into schizonts. Resulting merozoites are then released into the bloodstream to undergo asexual multiplication in red blood cells. A portion of these replicating asexual forms can differentiate into gametocytes which, following ingestion by blood-feeding female Anopheles mosquitoes, develop into sporozoites and are transmitted to another host via mosquito saliva. The genus Plasmodium currently comprises approximately 100 species that infect various mammals, birds and reptile hosts. Among these, six species commonly infect humans and are important causative agents of human malaria: P. falciparum, P. vivax, P. malariae, P. ovale curtisi, P. ovale wallikeri, and P. knowlesi. Despite an early observation of viral-like particles in cytoplasmic vacuoles of simian P. cynomolgi sporozoites [30], no viruses have been discovered in the parasites responsible for malaria.

With 219 million cases reported in 2017 in 90 countries around the world, malaria continues to be the most important protozoan disease affecting humans [31]. Despite ongoing and considerable global public health efforts, recent progress in reducing the disease burden due to malaria has stalled. Reasons include the emergence of resistance to insecticides in the mosquito vectors and parasite resistance to antimalarial drugs in humans. In addition, the large number of asymptomatic and/or submicroscopic Plasmodium infections in peripheral blood are an important source of transmission and pose a major challenge to control and eradication strategies [32]. This is compounded by the ability of some Plasmodium sp., including P. vivax, P. ovale curtisi, and P. ovale wallikeri, to form latent liver stages and later relapse. They also illustrate the need for approaches targeting the human parasite reservoir rather than treating only those with clinical disease.

There is an obvious interest in identifying viruses associated with human Plasmodium species from both an evolutionary and clinical perspective. The presence of RNA viruses infecting hematozoa parasites have been largely overlooked, although their position deep in the eukaryotic phylogeny means that they may constitute a valuable source of information to help understand early events in the evolution of eukaryotic RNA viruses. Knowledge of Plasmodium-specific viral infection may also provide insights into parasite biology in humans and mosquitoes, with the potential for identifying preventative or therapeutic strategies.

**Results**

**Plasmodium-infected human samples**

To investigate the virome of Plasmodium parasites that infect and cause disease in humans, we performed a meta-transcriptomic study of three species—P. vivax (hereby denoted Pv), P. knowlesi (Pk) and P. falciparum (Pf). These samples were obtained from 7, 6 and 5 malaria patients, respectively, at different locations in the state of Sabah, east Malaysia (S1 Table) [33]. All patients with malaria had uncomplicated disease. An additional library of 6 uninfected patients was also included as a negative control. All infected blood samples were validated for
their corresponding *Plasmodium* species (S1 Table). Microscopic parasite counts from peripheral blood films revealed similar densities (i.e. no significant differences, p-value = 0.7) between the three *Plasmodium* species, with parasitemia centered around 6000–8000 parasites per μL (S1 Fig).

**Sample processing**

Homogenous and equimolar ratios of each of the total RNA samples were used to prepare RNA-Seq libraries. Sequencing depth was similar for all samples, with 17±0.5 million reads obtained (Fig 1A). The human and ribosomal RNA (rRNA) read depletion drastically reduced the number of reads in both the non-infected and Pf data sets (93 and 81% of reads filtered, respectively) (Fig 1B) and to a lesser extent in Pk and Pv (only 42–57% of reads removed). Pf

![Figure 1A](https://doi.org/10.1371/journal.ppat.1008216.g001)

**Fig 1.** Host read depletion in RNA-Seq libraries. Reads were mapped against rRNA SILVAdb (SortMeRNA tool), the human genome, and the genomes of three *Plasmodium* species. (A) Efficiency of successive read filtering (rRNA and host sorting). (B) Proportion of major host transcripts in each data set. The number of reads mapping to the human genome, *Plasmodium* sp. genomes and MaRNAV-1 are expressed as the percentage of trimmed reads for each library.

https://doi.org/10.1371/journal.ppat.1008216.g001
transcripts were less abundant in libraries than those in Pk and Pv. Finally, the contig assemblies performed on each library depleted for rRNA, human and *Plasmodium* reads were almost equally successful for all libraries, with a similar contig length distribution between the data sets (S2 Fig).

**Virus discovery in *Plasmodium vivax*-infected blood samples**

**Discovery of a bi-segmented RNA Narna-like virus.** Ribosomal RNA-depleted data sets were submitted to BLASTx against a database containing all the RNA-dependent RNA polymerase (RdRp) protein sequences available at the NCBI. We focused on this protein as it is the mostly highly conserved among RNA viruses and hence constitutes the best marker for detecting their presence and performing expansive phylogenetic analyses. False-positive hits (i.e. non-viral contigs) were discarded by using a second round BLAST against the non-redundant protein (nr) database and removing contigs with non-viral top hits. Notably, true-positive RdRp signals were only found in the Pv library (Table 1).

These contigs all correspond to variants of the same gene from the Trinity assemblies, and all share their highest sequence similarity score (between 42.6 and 42.9% identity, Table 1) with the RdRp of Wilkie narna-like virus 1, an unclassified virus related to the narnaviruses recently identified in mosquito samples [34]. The mapping of Pv-reads against this newly-described viral-like genome revealed that the virus-like contig was highly abundant in the Pv library, comprising approximately 1.6% of all reads (from which rRNA has been excluded) (Fig 1). A more detailed characterization of this virus is presented below. To detect homologs to this newly identified RNA-virus-like contig in the other *Plasmodium* species, DN5867 contigs were used as the reference for another round of BLASTx: this analysis revealed no matches in either the Pf or Pk data sets.

The apparent bias in virus composition between libraries likely reflects differences in their virome composition rather than experimental bias, since the quality of samples, the depth of sequencing, and the contig assembly were similar among the four libraries. However, it is also possible that it in part reflects the limits of BLASTn/BLASTx sequence-based homology detection methods to identify highly divergent RNA viruses. To help overcome this limitation, and try to identify any highly divergent RNA viruses, we performed a BLASTn/BLASTx search on the contigs using the nt and nr databases, respectively. Assuming that RNA virus-like genomic sequence would be of a minimum length set to 1000 nt (as there are no RNA viruses shorter than this), and to make the analysis computationally tractable, only the “orphan” contigs (i.e. those without any match in any of the nt or nr databases) longer than 1000nt were used for further analysis (S3 Fig). To identify remote virus signal from these sequences, a second round of BLASTx search was conducted with lower levels of stringency: this revealed no clear hits to RNA viruses.

| Contig query | Length | estimated_count | BLASTn | BLASTx best hit | %ID | e-value | taxID | Virus              |
|--------------|--------|-----------------|--------|-----------------|-----|---------|-------|--------------------|
| Pv_1_DN5867_c0_g1_i1 | 2924   | 234605.7        | No hit | YP_009388589.1 RdRp | 42.8 | 1.30E-170 | 2010280 | Wilkie narna-like virus 1 |
| Pv_1_DN5867_c0_g1_i2 | 3023   | 77610.78        | No hit | YP_009388589.1 RdRp | 42.8 | 1.10E-170 | 2010280 | Wilkie narna-like virus 1 |
| Pv_1_DN5867_c0_g1_i3 | 3023   | 286828.4        | No hit | YP_009388589.1 RdRp | 42.6 | 3.70E-184 | 2010280 | Wilkie narna-like virus 1 |
| Pv_1_DN5867_c0_g1_i4 | 2141   | 105799.2        | No hit | YP_009388589.1 RdRp | 42.9 | 1.50E-185 | 2010280 | Wilkie narna-like virus 1 |
| Pv_1_DN5867_c0_g1_i5 | 3023   | 1834.39         | No hit | YP_009388589.1 RdRp | 42.8 | 1.10E-170 | 2010280 | Wilkie narna-like virus 1 |
| Pv_1_DN5867_c0_g1_i6 | 2045   | 79319.65        | No hit | YP_009388589.1 RdRp | 42.8 | 6.60E-171 | 2010280 | Wilkie narna-like virus 1 |
| Pv_1_DN5867_c0_g1_i7 | 1496   | 13751.44        | No hit | YP_009388589.1 RdRp | 42.8 | 8.30E-171 | 2010280 | Wilkie narna-like virus 1 |
Finally, we employed a structural-homology based approach to virus discovery, utilizing information on protein 3D structure rather than primary amino acid sequence, as the former can be safely assumed to be more conserved than the latter [35] and is therefore predicted to be better able to reveal distant evolutionary relationships. Accordingly, hypothetical ORFs were predicted from orphan contigs and Hidden Markov model (HMM) searches combined with 3D-structure modelling were performed on the corresponding amino acid sequences using the Phyre2 web portal [36]. Again, this revealed no reliable signal for the presence of highly divergent viruses in the RNA sequences obtained here.

Notably, with 122,452 reads mapped to it, the Pv unknown contig retained is highly expressed, possessing a similar level of abundance as the newly-identified Pv RdRp-like contig. Specifically, the abundance of these two contigs were in the same range, with Reads per Kilo-base per Million (RPKM) of the Pv RdRp-like and Pv unknown contigs of 2.9 and 2.6, respectively. Such a similarity in abundance levels supports the existence of a bi-segmented RNA virus. Finally, the 3kb RdRp-segment described in our _P. vivax_ samples is also within the range of the genome lengths seen in other members of the _Narnaviridae_ (2.3 to 3.6 kb).

**Narna-like virus genome and protein annotation.** The two segments of our putative narnavirus were both validated by Reverse Transcriptase-PCR (RT-PCR) in each of the seven _P. vivax_ samples used for this study, but were not found in the _P. knowlesi, P. falciparum_ nor the uninfected samples (Fig 2). Corresponding amplicons were then Sanger-sequenced to define both the inter-sample sequence diversity. We named this new virus Matryoshka RNA virus 1 (MaRNAV-1) because of its “Russian doll” composition, reflecting a virus that infects a parasite (protist) that infects an animal. The Sanger sequencing results of MaRNAV-1 for each Pv sample revealed a very high level of sequence conservation in the RdRp-encoding segment (“RdRp-segment”; Fig 3A, left).

Virus ORFs were predicted using the ORFinder NCBI tool and corresponding amino acid sequences were obtained and aligned (Fig 3A, right). This revealed that the nucleotide polymorphisms described above were also present at the amino acid level, even though these sequences were still highly conserved (98–100%), especially in the RdRp. An additional attempt at functional annotation was performed but did not reveal any additional functional motifs or domains aside from the RdRp.

The second segment, the presence of which distinguishes MaRNAV-1 virus from all other narnaviruses, was also highly conserved between _P. vivax_ samples (between 95 and 100% identity at the nucleotide level) and is likely to encode two protein products of 205 and 163 amino acids in length through two overlapping ORFs (Fig 3B). Unfortunately, the level of sequence divergence between this second segment and all other sequences available at NCBI meant that no functional annotations were possible.

Very few nucleotide polymorphisms were observed between the viruses identified from samples #1, #3, #4, #5, #6 and #10, which effectively showed 100% sequence identity (Fig 3C). In contrast, the MaRNAV-1 segments I and II from sample #2 are more distant, with 93% and 95% nucleotide identity, respectively, to the other sequences (Fig 3C) and 98% and 96% (ORF1) or 97% (ORF2) identity, respectively, at the amino acid level. Why sample #2 exhibits more diversity is unclear. It is similarly uncertain why segments I and II differ in levels of genetic diversity. Obtaining a satisfactory answer to this question will require more detailed information on protein function.

**Phylogenetic analysis of MaRNAV-1.** To link the newly-identified _Plasmodium vivax_ virus to the known diversity of RNA viruses, we performed a phylogenetic analysis with the sequence newly acquired here and the closest available relatives identified with BLASTx (Fig 4). To our knowledge, this is the first description of a virus associated with _Plasmodium_ spp. and few apicomplexan-related viruses have been isolated to date. Hence, it is not
Fig 2. RT-PCR confirmation of host and virus-like sequences in all *Plasmodium*-infected and non-infected samples used in this study. From left to right: RT-PCR of each of the samples using human RPS18 primers, *Plasmodium* LDHP primers, MaRNAV-1 Segment I (S1) primers and MaRNAV-1 Segment II (S2) primers. Numbers in parentheses correspond to the expected size of the corresponding amplicon. (*) indicate different parts of the same gel that have been cropped for ease of visualization only.

https://doi.org/10.1371/journal.ppat.1008216.g002
surprising that only low levels of amino acid sequence similarity (between 15 and 54%) were found in comparisons between MaRNAV-1 and the closest related RNA viruses available at NCBI. Importantly, however, the most closely related viruses were consistently classified as members of the family Narnaviridae (genus Narnavirus)—a group of single-stranded positive-sense RNA viruses known to infect such host species as fungi, plants and, importantly, protists (Fig 4). The most closely related virus—Wilkie narna-like virus 1—was recently identified in a

Fig 3. Genomic organization and sequence polymorphism of MaRNAV-1 in the seven P. vivax-infected blood samples. (A) RdRp-segment analysis. Left: Nucleotide sequence alignment and ORF prediction (orange boxes). Right: Protein sequence alignment and InterPro domains predicted (green boxes). (B) Nucleotide polymorphism and ORFs predicted from the segment II in P. vivax samples. Sequence polymorphisms are highlighted in black. (C) Distance matrix of segment I (left) and segment II (right) with percentage of identity obtained at the nucleotide level.

https://doi.org/10.1371/journal.ppat.1008216.g003
large-scale survey of mosquitoes [34] and is yet to be formally taxonomically assigned. Although the low abundance of this virus meant that no host could be conclusively assigned, a preliminary study suggested that it was unlikely to be a virus of mosquitoes, such that it could, in theory, infect a protozoan within the mosquitoes. In addition, two of the other narnaviruses most closely related to MaRNAV-1 virus—Leptomonas seymouri narna-like virus 1 (Lep-seyNLV1) and Phytophtora infestans RNA virus 4 (PiRV-4)—are seemingly associated with unicellular eukaryotes—Leptomonas seymouri and Phytophtora infestans, respectively [17, 37].

The second putative segment found in all the P. vivax samples described here also aligned with the second segment present in LepseyNLV1 which similarly encodes two overlapping ORFs (KU935605.1), even though they share little sequence similarity (only 14–18% identity at the amino acid level for ORF1 and ORF2, respectively). This high divergence explains why this sequence was not identified in previous BLASTx analyses and precluded more detailed phylogenetic analysis.

**Virus-host assignment**

A major challenge for all metagenomic studies is accurately assigning viruses to hosts as they could in reality be associated with host diet, the environment surrounding the sampling site, or a co-infecting micro-organism. In tentatively assigning hosts we assumed that: (i) a virus

---

Fig 4. Phylogenetic analysis of MaRNAV-1 associated with *Plasmodium vivax*. Boxes refer to the newly-described MaRNAV-1 viral sequences obtained in this study (red) or to RNA viruses classified as members of the *Narnaviridae* (dark orange), genus *Narnavirus* (light orange) or currently unclassified (grey). Taxa corresponding to the validated (coloured icons, right) and non-validated (grey icons, right) hosts are reported on the left part of the tree. Bootstrap values are indicated on each branch. The tree is mid-point rooted for clarity only.

https://doi.org/10.1371/journal.ppat.1008216.g004
with a high abundance is likely to be infecting a host found also in high abundance, (ii) a virus consistently found in association with one particular host is likely to infect that host, (iii) a virus that is phylogenetically related to those previously identified as infecting a particular host taxa is likely to infect a similar range of host taxa, and (iv) a virus and a host that share identical genetic code and/or similar codon usage or dinucleotide compositions are likely to have adapted and co-evolved, indicative of a host-parasite interaction.

**Eukaryotic host read profiling.** To initially assess whether MaRNAV-1 is likely to infect *Plasmodium* rather than other intra-host microbes and co-infecting parasites, the host taxonomy of the BLASTn/x top hits for each contig of the human and *Plasmodium*-depleted *P. vivax* library were compared to their respective size and abundance. However, this analysis revealed only a small number of short contigs associated with fungi and bacteria (S4 Fig). This result is of note as the usual hosts associated with narnaviruses are fungi, and the closest relatives have been found in mosquito samples, although the true host of this virus could in theory be protozoan. Among the Metazoa identified, all the contigs were associated with vertebrates, rather than members of the Arthropoda or Nematoda. Hence, *Plasmodium vivax* appears to be the most likely host of the newly-identified MaRNAV-1 virus.

**Comparison of codon usage and dinucleotide composition.** Host adaptation can result in specific patterns of codon and dinucleotide usage. We compared the codon usage observed in MaRNAV-1 to those of the potential host organisms. The Codon Adaptation Index (CAI) measures the similarity in synonymous codon usage between a gene and a reference set and was assessed for MaRNAV-1 using *H. sapiens, P. vivax* and *Anopheles* genera mosquitoes (pool of 7 species) as reference data sets. However, as none of the CAI/eCAI values obtained were significant (<1) (S5 Fig), no conclusions could be drawn regarding the potential host of MaRNAV-1. In a complementary approach, we compared the dinucleotide composition bias between the newly identified virus and the potential hosts [38]. Again, the dinucleotide frequencies in the two potential hosts *An. gambiae* and *P. vivax* revealed strong similarities that prevented us from identifying any signature of viral adaptation (S6 Fig).

**Investigation of the MaRNAV-1 and Plasmodium sp. association using the Sequence Read Archive (SRA).** To further test for an association between MaRNAV-1 and *Plasmodium* parasites, we performed a wider investigation of the occurrence of this virus in *Plasmodium*-infected samples and other *Plasmodium* species for which RNA-Seq data were available on the SRA. These data sets comprised *P. chabaudi, P. cynomolgi, P. falciparum, P. yoelli, P. knowlesi* and *P. berghei* (the relevant Bioprojects are listed in S2 Table). Reads counts <10 were considered too low to be informative.

In total, 1682 RNA-Seq data sets from *Plasmodium*-related projects on the SRA were screened for the presence of MaRNAV-1 using BLASTx. Reads mapping to MaRNAV-1 were identified in 45 libraries (including biological replicates), all of which belonged to *P. vivax* (Fig 5). Among the *P. vivax*-related runs (S3 Table), none of the 31 uninfected or *P. falciparum*-infected samples contained MaRNAV-1 (Chi-squared test, p-value = 0, S7A Fig). This pattern is strongly suggestive of a specific association between MaRNAV-1 and *P. vivax*, rather than the result of experimental bias or contamination introduced during RNA extraction or sequencing. Moreover, MaRNAV-1 was found in 43% (13 out of 30) of the *P. vivax*-infected SRA samples investigated here (biological replicates omitted). No obvious biological or experimental features were identified that could reasonable explain these patterns of prevalence.

In addition, the detection of MaRNAV-1 in the SRA-based studies was independent of the geographic location of where the *P. vivax* isolates were sampled (Colombia, Cambodia and Thailand) and the sample type obtained (human blood, mosquito dissected salivary glands or ex vivo cultures) (Chi-squared tests, p-values > 0.05, S7C and S7D Fig). Hence, together, these results strongly support a specific association between MaRNAV-1 and *P. vivax*. 

---

PLOS Pathogens | https://doi.org/10.1371/journal.ppat.1008216 December 30, 2019 10 / 26
Finally, we performed an additional screen of the MaRNAV-1 on 42 SRA libraries from *P. vivax*-free mosquitoes belonging to seven Anopheles species that are considered among the major vectors of *P. vivax* in the Asian Pacific region (S4 Table) [39]. Notably, no MaRNAV-1-like reads were identified in these data, strongly supporting the association of MaRNAV-1 with the presence of *P. vivax* and not the mosquito. These results are also consistent with the observation that all the three *Plasmodium* species tested in the study site of Sabah share the same predominant mosquito vector—*A. balabacensis*—yet MaRNAV-1 sequences were only found in *P. vivax* samples.

**Analysis of SRA-derived MaRNAV-1 virus-like genomes**

Narnavirus positive *P. vivax* data sets were further analyzed following the same workflow as described above. Hence, contigs were *de novo* assembled and re-submitted to BLASTx to extract full-length contigs corresponding to MaRNAV-1. The genomes obtained were
validated and quantified using read mapping and overlapping contigs were merged to obtain full-length viral genomes.

A phylogenetic analysis of these sequences containing MaRNAV-1 was performed at the nucleotide level (Fig 6). Importantly, phylogenetic position was strongly associated with sampling location rather than the nature of the samples (i.e. human-infected or mosquito-infected samples) are indicated on the right. Colored boxes indicate the samples collected in Asia (green), in South America (orange) or from unknown location (grey; ND: non-determined). The tree is mid-point rooted for clarity only.

https://doi.org/10.1371/journal.ppat.1008216.g006

**Detection of MaRNAV-2 in Leucocytozoon parasites infecting birds**

To investigate whether these narna-like sequences might infect a wider taxonomic distribution of hosts, we performed a complementary analysis of bird samples infected by members of the genus *Leucocytozoon*: apicomplexan parasites that belong to the same hematozoa subclass (of the Apicomplexa) as *Plasmodium*. These complementary studies were conducted on available RNA-Seq data previously obtained from liver, brain, heart and kidney tissues from Australian
Magpie, Pied currawong and Raven birds collected at various time points in New South Wales, Australia (S5 Table). Using the newly-discovered MaRNAV-1 viral segments as references, a BLASTx analysis was performed on RNA-Seq data previously obtained for these samples. A first segment encoding a single predicted ORF of 859 amino acid long and containing the RdRp domain motif was retrieved and compared to the *P. vivax* MaRNAV-1 sequences (S9A Fig). A relatively high level of sequence similarity (73% identity at the amino acid level) was observed between the *Leucocytozoon*-infected birds and the viral sequences found in the *P. vivax* infected-humans. A second segment was also extracted from these avian libraries that exhibited strong similarities in terms of length, genome organization and sequence identity with the prediction of two overlapping ORFs, denoted ORF1 and ORF2, that encode proteins of 246 and 198 amino acids, respectively, and that share 48–52% amino acid identity with the MaRNAV-1 segment II ORFs (S9B Fig). The relative abundance of the *Leucocytozoon* and both segments of MaRNAV-1 like transcripts were assessed in all the five RNA-Seq libraries by counting the total reads that mapped to the respective sequences using Bowtie2 and showed an overall positive correlation ($R^2 = 0.75$), even though discrepancies can be observed between the libraries (Fig 7).

Next, we explored the association between the presence of the *Leucocytozoon* parasites and the MaRNAV-1 virus homologs by performing RT-PCR on each individual sample previously used to perform RNA-Seq. The two viral segments were always found as both-present or both-absent for all of the 12 avian samples analyzed (S5 Table, S10 Fig). In addition, in the majority of samples (25 of 27), the presence of the viral segments was directly linked to the presence of the parasite: that is, the virus was present only when the parasite was detected and absent in parasite-free samples (S5 Table). This supports the idea that the viral sequences screened are infecting the *Leucocytozoon* parasite rather than the bird carrying it. Because of its similarity to *P. vivax* MaRNAV-1, we term this *Leucocytozoon* parasite MaRNAV-2.
MaRNAV-2 sequences were detected in 6 out of the 7 individual birds carrying the Leucocytozoon, independently of the tissue, date of sampling or bird species collected (Table S5). Interestingly, the only Leucocytozoon parasites free of MaRNAV-2 (sample 9585.3 collected from an Australian Magpie) may belong to a different Leucocytozoon species as it is phylogenetically distinct from the cluster formed by all the other Leucocytozoon populations in an analysis of the cytB gene (S11A Fig). A phylogenetic analysis of the Leucocytozoon MaRNAV-2 amino acid sequences revealed a strong clustering of the RdRp-segment with the P. vivax-infecting MaRNAV-1 viruses (Fig 8). Together, these Plasmodium and Leucocytozoon-associated viruses are likely to belong to a newly-described viral clade associated with haematozoa parasites. In addition, for both segment I (S11B Fig) and segment II (S11C Fig), the viral sequence variability between samples reflects the bird species rather than the location or the date of sampling. Interestingly, the overall level of divergence is similar between the two putative segments (between 86 and 100% identical nucleotide sites).

**Discussion**

Our meta-transcriptomic study of human blood samples infected with three major Plasmodium species revealed the presence of a highly abundant and geographically dispersed bi-segmented RNA virus in *P. vivax* that we named Matryoshka RNA virus 1 (MaRNAV-1). To the best of our knowledge this is the first documented RNA virus associated with the genus Plasmodium, and more broadly in parasites of the Apicomplexa subclass haematozoa. An additional investigation of complementary data sets from the SRA similarly provided strong evidence for the presence of MaRNAV-1 in *P. vivax*, but not in any of the other Plasmodium species.
analyzed. Similarly, MaRNAV-1 was absent from *Plasmodium*-free *Anopheles* species. Notably, MaRNAV-1 is both highly conserved and at high prevalence in *P. vivax* populations from both South East Asia and South America. Finally, we documented closely related viral sequences—MaRNAV-2—in avian samples infected with *Plasmodium*-related *Leucocytozoon* parasites. The divergent nature of the *Plasmodium* and *Leucocytozoon* viruses identified here, including the presence of a second genome segment, raises the possibility that they should be classified as a new genus or into a larger family together with LepseyNLV1.

The first segment of MaRNAV-1 encodes a single ORF containing the conserved RdRp-motif that is related to those found in the *Narnaviridae*, while the second segment, which is not characteristic of narnaviruses, encodes two overlapping ORFs of unknown function. The family *Narnaviridae* comprises a capsid-less viral family that infects plants, fungi and protists. Interestingly, no sequences associated with fungi were observed in our samples, again compatible with the idea that this virus is indeed associated with *Plasmodium*. In addition, the closest RNA virus homologs were also observed in protozoans, or in arthropods that could conceivably be infected by protozoan parasites [34, 40]. This virus-protoist association evidence was reinforced by the consistent link between this virus and *P. vivax*, rather than to the metazoan hosts (mosquitoes and human) from which the samples were extracted, or the other *Plasmodium* species, including its absence in *Plasmodium*-free *Anopheles*.

The evolutionary origin of these novel *Plasmodium* and *Leucocytozoon* viruses is less clear, but can be framed as two hypotheses: (i) an ancient virus-host co-divergence between the Euglenozoa (e.g. *Leptomonas*) and Alveolate (including the hematozoa) groups of eukaryotes at almost two billion years ago [41] (Fig 9A), or (ii) horizontal virus transfer events between either a secondary (likely invertebrate) host and protozoan parasites, or among two protozoan parasites co-infecting the same secondary host, over an unknown time-scale (Fig 9B). As RNA viruses normally exhibit high rates of evolutionary change [42], the recognizable sequence similarity between the narna-like RNA viruses from *Plasmodium* (Alveolates, Apicomplexa) and *Leptomonas* (Euglenozoa, kinetoplastids) means it is perhaps unlikely that they shared a common ancestor that lived approximately two billion years ago (Figs 8B and 9). Some Euglenozoa and Alveolates independently evolved a parasitic lifestyle by infecting invertebrates and, more recently, vertebrate hosts. Hence, it is more likely that the protozoan narnavirus-like similarities reflect viral cross-species transmission between two protozoan parasites during mixed-infection in either vertebrate or invertebrate hosts (Fig 9B). The wide distribution and prevalence of MaRNAV-1 in *P. vivax* populations, as well as in the different species of *Leishmania* parasites investigated previously, supports the idea that this host jumping event is relatively ancient, although the exact time-scale is difficult to determine. As previously demonstrated, invertebrates play a key role in RNA virus evolution by feeding on many different hosts and transmitting viruses, fungi and protozoa among both plants and vertebrates [41, 43]. This may also explain why narnaviruses or closely related RNA virus have been able to spread to such a diverse range of eukaryotes, including Fungi, Stramenopiles, Alveolates and Euglenozoa. Moreover, the recent characterization of a narnavirus in the plant-infecting trypanosomatid *Phytomonas serpens* [44] suggests that vertebrates are not likely to be the hosts where the horizontal virus transfer occurred.

The RdRp segment of MaRNAV-1 documented here is clearly related to the narnavirus RdRp, although we are unable to identify a clear homolog for the second, divergent segment. Hence, as previously hypothesized for the tri-segmented plant RNA virus outermiviruses that combine a *Narnaviridae*-like RdRp and *Tomusviridae*-like movement and capsid proteins [45], our newly-described viruses may have evolved by reassortment of different RNA viruses during co-infection, resulting in the combination of RdRp from Narnavirus and another two ORFs from an undescribed yet RNA virus family or families (Fig 9B). Further investigation of
The origins and functions of these hypothetical proteins clearly need to be conducted to better understand the virus biological cycle and its evolutionary history. Indeed, capsid-less elements cannot exist in an extracellular state and are necessarily transmitted via intracellular mode (cell division or cell mating) [46, 47].

The Narnaviridae comprise some of the simplest viruses described to date, containing a single segment encoding a single replicase. Despite this, they are still able to impact their hosts in profound ways. For example, a reduction in host virulence (hypovirulence) has been documented in the case of mitoviruses (a genus of Narnaviridae infecting fungi) [47]. Combined with the previously reported impacts of dsRNA viral infections on the biology, pathogenesis

---

**Fig 9. Hypothetical scenarios for the origin and evolution of MaRNAV-1 and MaRNAV-2 and relatives among parasites belonging to the Alveolates and Euglenozoa eukaryotic groups.** (A) Ancient virus-host co-divergence between the Euglenozoa and Alveolates that may have occurred approximately two billion years ago. (B) Horizontal virus transfer between the Alveolates and Euglenozoa parasites that co-infected the same secondary (likely invertebrate) host.

https://doi.org/10.1371/journal.ppat.1008216.g009
and treatment response of the human parasite *Leishmania* [20], understanding effects of on *P. vivax* biology and pathogenesis is clearly an area of interest, although this will require confirmation of the replicative activity of the newly discovered viruses in *Plasmodium* as well as a greater understanding of the biology of infection. Intuitively, the biological consequences of the high prevalence of this virus in *P. vivax*-infected individuals will represent an important avenue for future research. More broadly, the characterization of the viral cycle of MaRNAV-1, their biology, and interactions with its host may also help to understand the biology and life-cycle of *P. vivax* parasites, as well as the modulation of host and parasite responses leading to immunoevasion, pathogenesis and transmission. Similarly, additional study of those *Pv*-infected samples that do not carry MaRNAV-1 may reveal some of the possible impacts of viral infection on parasite biology. Future work should also focus on the impact of virus infection on the hypnozoite liver stage of *P. vivax*, which is not present among the other, virus-negative, *Plasmodium* species assessed in this study, and is responsible for *P. vivax* infection relapses in human hosts and ongoing transmission in the absence of specific liver treatment. Finally, it will be important to determine whether MaRNAV-1 or a related virus infects the other human *Plasmodium* spp. with the hypnozoite life-cycle stage—*P. ovale curtisi* and *P. ovale wallikeri* [48]—as well as the possible role of viral infection in promoting immunoevasion, such as asymptomatic infection [49] or pathogenesis [50]. Most of the samples tested here correspond to symptomatic forms of *Plasmodium* infection, and it is possible that different virus-parasite interactions are seen in asymptomatic cases.

**Materials and methods**

**Ethics statement**

The study was approved by the ethics committees of the Malaysian Ministry of Health (NMRR-10-754-6684) and Menzies School of Health Research (HREC 2010–1431). All adults provided written informed consent, including the parents or guardians of children aged less than 18-years.

**Biological samples**

Whole blood samples (1 ml) were collected at district hospitals from healthy and *Plasmodium*-infected patients in the state of Sabah, east Malaysia in 2013–14. Patients had a clinical illness consistent with malaria, with blood collected prior to antimalarial treatment. Parasite density was quantified by research microscopy using pre-treatment slides and reported as the number of parasites per 200 leukocytes from thick blood film. This was converted into the number of parasites per microliter using the patient's leukocyte count from their hospital automated hematology result. Remaining blood samples were stored in RNAlater and conserved at -80°C until RNA extraction. Sampling locations, sampling dates, *Plasmodium* species validation and parasite counts are reported in S1 Table.

PCR validation for *P. vivax* and *P. falciparum* were conducted following Padley et al. [51]. A single-round PCR was performed using one single reverse primer in combination with species-specific forward primers (S6 Table). The *P. knowlesi*-infected sample validation were conducted following Imwong et al. [52] using a nested-PCR strategy with two primer couples: rPLU3 and rPLU4 for the first PCR, and PfF1140 and PfR1150 for the nested PCR (see S6 Table for the corresponding sequences).

**Total RNA extraction and RNA sequencing**

Total RNAs were extracted from blood samples using the Qiagen RNeasy Plus Universal MIDI kit and following manufacturer’s instructions. Importantly, randomized and serial extractions
were conducted to prevent potential experimental biases and to facilitate the detection of kit, columns, reagents or extraction-specific contamination from the corresponding meta-trascriptomic data.

Total RNA samples were grouped by *Plasmodium* species and pooled in equimolar ratios into a single sample. Quality assessments were then conducted and four TruSeq stranded libraries were synthetized by the Australian Genome Research facility (AGRF), including a rRNA and globin mRNA depletion using RiboZero and globin depletion kit from Illumina. Resulting libraries were run on HiSeq2500 (paired-end, 100bp) platform at the AGRF (RNA sample quality and the features of each library are described in S7 Table).

**rRNA and host read depletion**

Raw reads were first trimmed using the Trimmomatic software [53] to remove Illumina adapters and low-quality bases. Human, rRNA and *Plasmodium* associated reads were removed from the data sets by successively mapping the trimmed reads to the latest versions of each corresponding reference sequence databases (see S8 Table for more details) with either SortmeRNA [54] or Bowtie2 software, and by applying local and very-sensitive options for the alignment [55]. All corresponding databases and the software used for the host analyses and rRNA depletions are summarized in S8 Table.

**Contig assembly and counting**

Depleted read data sets were assembled into longer contigs using the Trinity software [56]. The resulting contig abundances were estimated using the RSEM software [57].

**Virus discovery**

A global sequence-based homology detection was performed using BLASTn and Diamond BLASTx [58] against the entire non-redundant nucleotide (nt) or protein (nr) databases with using e-values of 1e-10 and 1e-5, respectively. Profiling plots were obtained by clustering contigs based on the taxonomy of their best BLASTn and/or BLASTx hits (highest BLAST score) and plotting their respective length and abundance using ggplot2 [59].

In parallel, a RNA virus-specific sequence-based homology detection was conducted by first aligning our data sets to a viral RdRp database using Diamond BLASTx. To ensure the removal of false-positives, a second BLASTx round using exhaustive hits output parameters was performed on each RdRp-matched contig to discard those that are more likely from a non-viral source. True-positive viral contigs were merged when possible and further analyzed using Geneious 11.1.4 software [60].

“Unknown contigs” (i.e. contigs with no BLASTx hit) longer than 1kb were retained and submitted to a second round of BLASTx using a low-stringent cut-off of 1e-4. HMM-profile and structural-based homology searches were also performed on these unknown contigs using the normal mode search of the Protein Homology/analogY Recognition Engine v 2.0 (Phyre2) web portal [36]. Briefly, the amino acid sequences of predicted ORFs were first compared to a curated non-redundant nr20 data set (comprising only sequences with <20% mutual identity) using HHblits [61]. Secondary structures were predicted from the multiple sequence alignment and this information was converted into a Hidden Markov model (HMM). This HMM was then used as a query against a HMM database built from proteins of known 3D-structures and using HHsearch [62]. Finally, a 3D-structure modelling step was performed using HHsearch hits as templates, following the method described in [61].

Virus-like sequences were further experimentally confirmed in total RNA samples by performing cDNA synthesis using the SuperScript IV reverse transcriptase (Invitrogen, Catalog
number: 11756500) and PCR amplification with virus candidate specific primers using the Platinum SuperFi DNA polymerase (Invitrogen, Catalog number: 12359010). Amplified products were Sanger sequenced using intermediary primers enabling a full-length coverage (all primers are listed in S6 Table).

**Host-virus assignment**

To help assign a virus to a specific host (i.e. determining which host organism these viruses likely infect), we analyzed both codon usage bias and genomic dinucleotide composition [38, 63]. Accordingly, the average codon usage of *H. sapiens* and *P. vivax* were retrieved from the Codon Usage Database (available at http://www.kazusa.or.jp/codon/) and the codon adaptation index (CAI) and associated expected-CAI (eCAI) were determined using the CAIcal webserver, available at http://genomes.urv.es/CAIcal/E-CAI/ [64]. As the most prevalent *Anopheles* mosquito vector in the Sabah region of Malaysia (*Anopheles balabacensis*) did not have a codon usage table available, in this case we retrieved the codon usage from seven other *Anopheles* species (*A. dirus*, *A. minimus*, *A. cracens*, *A. gambiae*, *A. culicifacies*, *A. merus* and *A. stephensi*) which included major vectors of malaria in South East Asia.

As well as codon bias, we determined the dinucleotide composition of MaRNAV-1 and compared to that of *Anopheles gambiae* (RefSeq | GCF_000005575.2) for which a full-genome sequence is available, and *P. vivax* (RefSeq | GCF_000002415.2). The match between host and virus was then calculated using the method described previously [38] by calculating the $f_{xy}/f_x f_y$ ratio from the MaRNAV-1 sequences obtained by Sanger sequencing (see above).

**Virus genome characterization**

Validated virus-like genomes were further characterized using both genome/protein annotation programs, including InterProScan for protein domain, Sigcleave and Fuzzpro from EMBOSS package for signal cleavage sites and motifs, and TMHMM for transmembrane regions [65, 66].

**Mining of the Sequence Read Archive (SRA)**

To identify homologs of MaRNAV-1, the newly identified Narna-like virus sequence was used as a reference in both Magic-BLAST BLASTn (default parameters) [67] and Diamond BLASTx (cut-off 1e-5) [58] analyses of several RNA-seq data sets obtained from *Plasmodium sp.* available on the NCBI SRA using the NCBI SRA toolkit v2.9.2. The list of the corresponding BioProjects, runs and references are provided in S2 Table.

*P. vivax* SRA library information (i.e. host, location and biological replicates) was manually retrieved from the corresponding papers (S3 Table). Where possible, this information was used to assess whether such variables were associated with the detection of narna-like viruses by performing Chi-squared tests using the SPSS Statistics software (IBM). SRA runs positive for homologs to MaRNAV-1 (number of read BLAST hits >100) were imported locally and assembled following the same workflow as previously used to extract homologous full-length contigs and to calculate their relative abundance in the samples.

To further assess host assignments, the same SRA data sets were subjected to Magic-BLAST using *Plasmodium* and mosquito and human specific housekeeping gene transcripts (LDH-P gb|M93720.1 and RSP-7 gb|L20837.1, respectively). This large-scale analysis may necessarily result in false-negative results because of the idiosyncrasies of the experimental procedures used, such as the depletion of human reads or *Plasmodium* RNA enrichment, both of which can bias such host read counting. Moreover, some samples come from the same biological replicate and hence cannot be counted as independent. Such potential biases
forced us to manually retrieve all information for each *P. vivax* SRA library using related publications (S3 Table).

**Phylogenetic analysis**

Predicted ORFs containing the viral RNA-dependent (RdRp) domain from both the SRA and human blood and bird associated sequences (see below) were translated and aligned using the E-INS-I algorithm in MAFFT v7.309 [68]. To place the newly identified viruses into a more expansive phylogeny of RNA viruses, reference protein sequences of the closest homologous viral families or genera were retrieved from NCBI and incorporated to the amino acid sequence alignment. The alignments were then trimmed with Gblocks under the lowest stringency parameters [69]. The best-fit amino acid substitution models were then inferred from each curated protein alignment using either the Smart model selection (SMS) [70] or ModelFinder [71], and maximum likelihood phylogenetic trees were then estimated with either PhyML [72] or IQ-tree [73] with bootstrapping (1000 replicates) used to assess node support. For clarity, all phylogenetic trees were midpoint rooted.

**Analysis of avian meta-transcriptomic libraries**

To supplement our analysis of human *Plasmodium* samples, we analyzed four meta-transcriptomic libraries sampled from four bird species (*Gymnorhina tibicen*, *Strepera graculina*, *Corvus coronoides* and *Grallina cyanoleuca*) in New South Wales, Australia. Sampling details are reported in S5 Table. The RNA-Seq data analysis and the BLASTx detection of MaRNAV-1 homologs from bird sample data sets were conducted as described above.

The PCR-based detection of both narna-like viruses and *Leucocytozoon* parasites were conducted using newly-designed primers corresponding to the *Leucocytozoon* homologs of the MaRNAV-1 RdRp and segment II (primers are described in S6 Table), and following the same PCR protocol as described above. PCR-based *Leucocytozoon* detections were performed using primers targeting the *Leucocytozoon* mitochondrial cytochrome B oxidase gene as described in [74]. All additional analyses of the bird data sets were performed utilizing the software and tools described above.

**Supporting information**

**S1 Table. Description of human blood samples used in this study.** PCR: PCR-based validation of *Plasmodium* species using species-specific primers: Pv—*P. vivax*; Pk—*P. knowlesi*; Pf—*P. falciparum*; pc—parasite counting (i.e. parasite density / μL = number of parasites counted x patient’s lab leukocyte result / 200 leukocytes counted).

(DOCX)

**S2 Table. BioProject and corresponding SRA accessions used in this study.**

(DOCX)

**S3 Table. *Plasmodium vivax* SRA libraries.** Libraries positive for MaRNAV-1 are shown in red and those used for phylogenetic analysis (MaRNAV-1-like read counts > 100) are shown in bold. *Plasmodium*-free libraries are in grey. *Homo sapiens* = human blood samples. *Anopheles dirus* = mosquito salivary gland dissection samples. *Homo sapiens (ex vivo)*: Micropatterned cellular co-cultures. ND: Not determined.

(DOCX)

**S4 Table. BioProject and corresponding SRA accessions of *P. vivax*-free *Anopheles* species.**

(DOCX)
S5 Table. Bird sample analysis summary table. Presence, absence or uncertainty of *Leucocytozoon* detection from pathology reports are highlighted in green, red and orange, respectively. Positive or negative PCR targeting either the *Leucocytozoon* parasite, the RdRP-like segment and the unknown second segment of MaRNAV-2 are highlighted in green and red, respectively.

(DOCX)

S6 Table. Primers used in this study.

(DOCX)

S7 Table. Quality of RNA extraction and RNA-seq data sets obtained.

(DOCX)

S8 Table. List of databases and software used for rRNA and host read depletion.

(DOCX)

S1 Fig. *Plasmodium* parasite count in human blood samples. Parasite counts are expressed as the number of parasites per μl of blood.

(TIF)

S2 Fig. Results of the Trinity sequence assembly. Contig length and count obtained after performing Trinity assembly of libraries depleted in rRNA, human and *Plasmodium* reads.

(TIF)

S3 Fig. Orphan contig length and abundance. An arbitrary cut-off of 1000 nt was used to identify candidate RNA viruses. Abundance is expressed using the expected count value provided by the RSEM analysis.

(TIF)

S4 Fig. Length and abundance of contigs from candidate non-major host taxa (BLASTn/BLASTx). Abundance is expressed using the expected count value provided by the RSEM analysis.

(TIF)

S5 Fig. Comparison of CAI values obtained by comparing codon usage of MaRNAV-1 viral contigs retrieved from each *Plasmodium* library to the potential hosts *P. vivax*, *H. sapiens* and mosquitoes of the genus *Anopheles*.

(TIF)

S6 Fig. Odds ratios of dinucleotides (fxy/ffxy) from MaRNAV-1 contigs (bottom) versus *P. vivax* (top, right) and *A. gambiae* (top, left) genomic sequence.

(TIF)

S7 Fig. Association study between MaRNAV-1 contigs and the characteristics of *P. vivax* libraries at the SRA (Chi-squared tests). (A) *Plasmodium* infection association test. (B) Biological replicates association test. Replicates corresponding to the same biological sample are grouped by colour. (C) Sample location association test. (D) Host association test.

(TIF)

S8 Fig. Read count mapping to the MaRNAV-1 segment I (x-axis, log scaled) and Segment II (y-axis, log scaled) in *P. vivax* SRA data sets. The R-squared value is indicated on the right.

(TIF)

S9 Fig. Sequence alignment (MAFFT) of MaRNAV-2 contigs in *Leucocytozoon*-infected bird and MaRNAV-1 sequences in *P. vivax*-infected humans. (A) Analysis of segment I. (B)
Analysis of segment II. Nucleotide alignments are shown on the left and protein alignments are shown on the right (top—ORF1; bottom—ORF2). Orange boxes: predicted ORF using standard genetic code. Light green boxes: InterProScan domain prediction. Yellow to green plots: level of sequence conservation.

S10 Fig. PCR-based detection of leucocytozoons and MaRNAV-2 (2 segments) in avian cDNA samples. Top: *Leucocytozoon* CytB PCR; Middle: MaRNAV-2 segment I homolog detection; Bottom: MaRNAV-2 segment II homolog detection.

S11 Fig. Maximum likelihood phylogenetic trees of parasites and MaRNAV-2 homologs obtained from bird samples. (A) Hematozoa CytB phylogeny. The CytB sequence from *Gallus gallus* is used as an outgroup. Samples positive for MaRNAV-2 I are marked with *. (B) MaRNAV-2 segment I phylogeny; (C) MaRNAV-2 segment II phylogeny. Sequences from bird samples are shown in red.

S1 References. Supporting references.

Acknowledgments

We acknowledge the Sydney Informatics Hub and the University of Sydney’s high performance cluster Artemis for providing the computational resources required for the RNA-Seq data processing, and Wei-Shan Chang for providing technical assistance with PCR validation. We thank Dr. Andrew Harmon and Heeva Baharlou (Westmead Institute for Medical Research) for valuable input. We also thank the Director-General, Ministry of Health, Malaysia, for permission to publish this manuscript.

Author Contributions

Conceptualization: Justine Charon, Matthew J. Grigg, John-Sebastian Eden, Miles P. Davenport, Nicholas M. Anstey, Edward C. Holmes.

Data curation: Justine Charon, John-Sebastian Eden.

Formal analysis: Justine Charon, Hafsa Rana, Edward C. Holmes.

Funding acquisition: Matthew J. Grigg, Nicholas M. Anstey, Edward C. Holmes.

Investigation: Justine Charon, Hafsa Rana, Karrie Rose.

Methodology: Justine Charon, Matthew J. Grigg, John-Sebastian Eden, Kim A. Piera, Timothy William, Karrie Rose, Miles P. Davenport, Nicholas M. Anstey, Edward C. Holmes.

Resources: Matthew J. Grigg, John-Sebastian Eden, Kim A. Piera, Timothy William, Karrie Rose.

Supervision: Edward C. Holmes.

Validation: Justine Charon, Matthew J. Grigg, John-Sebastian Eden, Edward C. Holmes.

Visualization: Justine Charon, Edward C. Holmes.

Writing – original draft: Justine Charon.
Writing – review & editing: Matthew J. Grigg, John-Sebastian Eden, Kim A. Piera, Hafsa Rana, Miles P. Davenport, Nicholas M. Anstey, Edward C. Holmes.

References

1. Forterre P. Defining life: the virus viewpoint. Orig Life Evol Biosph. 2010; 40: 151–160. https://doi.org/10.1007/s11084-010-9194-1 PMID: 20198436

2. Angly FE, Felts B, Breitbart M, Salamon P, Edwards RA, Carlson C, et al. The marine viromes of four oceanic regions. PLoS Biol. 2006; 4: e368. https://doi.org/10.1371/journal.pbio.0040368 PMID: 17090214

3. Culley AI, Lang AS, Suttle CA. Metagenomic analysis of coastal RNA virus communities. Science. 2006; 312: 1795–1798. https://doi.org/10.1126/science.1127404 PMID: 16794078

4. Desnues C, Rodriguez-Brito B, Rayhaw S, Kelley S, Tran T, Haynes M, et al. Biodiversity and biogeography of phages in modern stromatolites and thrombolites. Nature. 2008; 452: 340–343. https://doi.org/10.1038/nature06735 PMID: 18311127

5. Paez-Espino D, Eloe-Fadrosh EA, Pavlopoulos GA, Thomas AD, Huntemann M, Mikhailova N, et al. Uncovering Earth’s virome. Nature. 2016; 536: 425–430. https://doi.org/10.1038/nature19094 PMID: 27533034

6. Suttle CA. Viruses in the sea. Nature. 2005; 437: 356–361. https://doi.org/10.1038/nature04160 PMID: 16163346

7. Zhang Y-Z, Shi M, Holmes EC. Using metagenomics to characterize an expanding virosphere. Cell. 2018; 172: 1168–1172. https://doi.org/10.1016/j.cell.2018.02.043 PMID: 29522738

8. Miles MA. Viruses of parasitic protozoa. Parasitol Today. 1988; 4: 289–290. https://doi.org/10.1016/0169-4758(88)90023-3 PMID: 15463003

9. Khramtsov NV, Woods KM, Nesterenko MV, Dykstra CC, Upton SJ. Virus-like, double-stranded RNAs in the parasitic protozoan Cryptosporidium parvum. Mol Microbiol. 1997; 26: 289–300. https://doi.org/10.1046/j.1365-2958.1997.5721933.x PMID: 9383154

10. Miller RL, Wang AL, Wang CC. Purification and characterization of the Giardia lamblia double-stranded RNA virus. Mol Biochem Parasitol. 1988; 28: 169–195. https://doi.org/10.1016/0166-6851(88)90003-5 PMID: 3398960

11. Tarr PJ, Aline RF Jr, Smiley BL, Scholler J, Keithly J, Stuart K. LR1: a candidate RNA virus of Leishmania. Proc Natl Acad Sci USA. 1988; 85: 9572–9575. https://doi.org/10.1073/pnas.85.24.9572 PMID: 3200841

12. Wang AL, Wang CC. A linear double-stranded RNA in Trichomonas vaginalis. J Biol Chem. 1985; 260: 3697–3702. PMID: 2982674

13. Wang AL, Wang CC. Discovery of a specific double-stranded RNA virus in Giardia lamblia. Mol Biochem Parasitol. 1986; 21: 269–276. https://doi.org/10.1016/0166-6851(86)90035-5 PMID: 3807947

14. Widmer G, Comeau AM, Furlong DB, Wirth DF, Patterson JL. Characterization of a RNA virus from the parasite Leishmania. Proc Natl Acad Sci USA. 1989; 86: 5979–5982. https://doi.org/10.1073/pnas.86.15.5979 PMID: 2762308

15. Akopyants NS, Lye L-F, Dobson DE, Lukeš J, Beverley SM. A novel bunyavirus-like virus of trypanosomatid protist parasites. Genome Announce. 2016; 4: e00715–16. https://doi.org/10.1128/genomeA.00715-16 PMID: 27491985

16. Grybchuk D, Akopyants NS, Kostygov AY, Konovalovas A, Lye L-F, Dobson DE, et al. Viral discovery and diversity in trypanosomatid protozoa with a focus on relatives of the human parasite. Proc Natl Acad Sci USA. 2018; 115: E506–E515. https://doi.org/10.1073/pnas.1717806115 PMID: 29284754

17. Lye L-F, Akopyants NS, Dobson DE, Beverley SM. A narnavirus-like element from the trypanosomatid protozoan parasite Leptomonas seymouri. Genome Announce. 2016; 4: e00713–16. https://doi.org/10.1128/genomeA.00713-16 PMID: 27491984

18. Sukla S, Roy S, Sundar S, Biswas S. Leptomonas seymouri narna-like virus and not leishmania-viruses detected in kala-azar samples from India. Arch Virol. 2017; 162: 3827–3835. https://doi.org/10.1007/s00705-017-3559-y PMID: 28939968

19. Padma TV. Russian doll disease is a virus inside a parasite inside a fly. New Scientist. August 10th, 2015. https://www.newscientist.com/article/dn28020-russian-doll-disease-is-a-virus-inside-a-parasite-inside-a-fly/.

20. Gómez-Arreaza A, Haenni A-L, Dunia I, Avián L. Viruses of parasites as actors in the parasite-host relationship: A “ménage à trois”. Acta Tropica. 2017; 166:126–132. https://doi.org/10.1016/j.actatropica.2016.11.028 PMID: 27876650
21. Fichorova RN, Lee Y, Yamamoto HS, Takagi Y, Hayes GR, Goodman RP, et al. Endobiont viruses sensed by the human host—beyond conventional antiparasitic therapy. PLoS One. 2012; 7: e48418. https://doi.org/10.1371/journal.pone.0048418 PMID: 23144874

22. Ito MM, Catanhêde LM, Katsuragawa TH, da Silva CF Junior, Camargo LMA, de Godoi Mattos R, et al. Correlation between presence of Leishmania RNA virus 1 and clinical characteristics of nasal mucosal leishmaniasis. Braz J Otorhinol. 2015; 81: 533–540. https://doi.org/10.1016/j.bjor.2015.07.014 PMID: 26277588

23. Ives A, Ronet C, Prevel F, Ruzzante G, Fuertes-Marraco S, Schutz F, et al. Leishmania RNA virus controls the severity of mucocutaneous leishmaniasis. Science. 2011; 331: 775–778. https://doi.org/10.1126/science.1199326 PMID: 21311023

24. Brettmann EA, Shaik JS, Zanger H, Lye L-F, Kuhlmann FM, Akopyants NS, et al. Tilting the balance between RNA interference and replication eradicates Leishmania RNA virus 1 and mitigates the inflammatory response. Proc Natl Acad Sci USA. 2016; 113: 11958–12005. https://doi.org/10.1073/pnas.1615085115 PMID: 27790961

25. Zanger H, Halli A, Despods C, Lye L-F, Akopyants NS, Dobson DE, et al. Leishmania aethiopica field isolates bearing an endosymbiotic dsRNA virus induce pro-inflammatory cytokine response. PLoS Negl Trop Dis. 2014; 8: e2836. https://doi.org/10.1371/journal.pntd.0002836 PMID: 24762979

26. Adali V, Lye L-F, Akopyants NS, Zimic M, Llanos-Cuentas A, Garcia L, et al. Association of the endobiont double-stranded RNA virus LRV1 with treatment failure for human Leishmaniasis caused by Leishmania braziliensis in Peru and Bolivia. J Infect Dis. 2016; 213: 112–121. https://doi.org/10.1093/infdis/jiv335 PMID: 26123565

27. Bourreau E, Ginouves M, Prévot E, Hartley M-A, Gangneux J-P, Robert-Gangneux F, et al. Presence of Leishmania RNA Virus 1 in Leishmania guyanensis increases the risk of first-line treatment failure and symptomatic relapse. J Infect Dis. 2016; 213: 105–111. https://doi.org/10.1093/infdis/jiv335 PMID: 26123564

28. Nilbert ML, Woods KM, Upton SJ, Ghabrial SA. Cryspovirus: a new genus of protozoan viruses in the family Partitiviridae. Arch Virol. 2009; 154: 1959–1963. https://doi.org/10.1007/s00705-009-0513-7 PMID: 19856142

29. Jenkins MC, Higgins J, Abrahante JE, Kniel KE, O'Brien C, Trout J, et al. Fecundity of vectors of human malaria in the Asia-Pacific region: occurrence data, distribution maps and bionomic precis. Parasit Vectors. 2011; 4: 89. https://doi.org/10.1186/1756-3305-4-89 PMID: 21162587
40. Shi M, Lin X-D, Tian J-H, Chen L-J, Chen X, Li C-X, et al. Redefining the invertebrate RNA virosphere. Nature. 2016; 540: 539–543. https://doi.org/10.1038/nature20167 PMID: 27880757

41. Hedges SB, Blair JE, Venturi ML, Shoe JL. A molecular timescale of eukaryote evolution and the rise of complex multicellular life. BMC Evol Biol. 2004; 4: 2. https://doi.org/10.1186/1471-2148-4-2 PMID: 15005799

42. Duffy S, Shackelton LA, Holmes EC. Rates of evolutionary change in viruses: patterns and determinants. Nat Rev Genet. 2008; 9: 267–276. https://doi.org/10.1038/nrg2332 PMID: 18319742

43. Li C-X, Shi M, Tian J-H, Lin X-D, Kang Y-J, Chen L-J, et al. Unprecedented genomic diversity of RNA viruses in arthropods reveals the ancestry of negative-sense RNA viruses. eLife. 2015; 4: e05378. https://doi.org/10.7554/eLife.05378 PMID: 25633976

44. Akopyants NS, Lye L-F, Dobson DE, Lukesˇ J, Beverley SM. A narnavirus in the trypanosomatid protist plant pathogen Phytomonas serpens. Genome Announce. 2016; 4: e00711-16. https://doi.org/10.1128/genomeA.00711-16 PMID: 27469953

45. Rastgou M, Habibi MK, Izadpanah K, Masenga V, Milne RG, Wolf YI, et al. Molecular characterization of the plant virus genus Ourmiavirus and evidence of inter-kingdom reassortment of viral genome segments as its possible route of origin. J Gen Virol. 2009; 90: 2525–2535. https://doi.org/10.1099/vir.0.013086-0 PMID: 19535502

46. Dolja VV, Koonin EV. Capsid-less RNA viruses. eLS. 2012; https://doi.org/10.1002/9780470015902.a0023269

47. Hillman BI, Cai G. The family Narnaviridae: simplest of RNA viruses. Adv Virus Res. 2013; 86:149–176. https://doi.org/10.1016/B978-0-12-394315-6.00006-4 PMID: 23498906

48. White NJ. Why do some primate malarias relapse? Trends Parasitol. 2016; 32: 918–920. https://doi.org/10.1016/j.pt.2016.08.014 PMID: 27743866

49. Padley D, Moody AH, Chiodini PL, Saldanha J. Use of a rapid, single-round, multiplex PCR to detect malarial parasites and identify the species present. Ann Trop Med Parasitol. 2003; 97: 131–137. https://doi.org/10.1179/000349803125002977 PMID: 12803868

50. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014; 30: 2114–2120. https://doi.org/10.1093/bioinformatics/btu170 PMID: 24695404

51. Remmert M, Biegert A, Hauser A, Söding J. HHblits: lightning-fast iterative protein sequence searching by HMM-HMM alignment. Nat Meth. 2012; 9: 173–175. https://doi.org/10.1038/nmeth.1818 PMID: 22198341
62. Söding J. Protein homology detection by HMM-HMM comparison. Bioinformatics. 2005; 21: 951–960. https://doi.org/10.1093/bioinformatics/bti125 PMID: 15531603

63. Su M-W, Lin H-M, Yuan HS, Chu W-C. Categorizing host-dependent RNA viruses by principal component analysis of their codon usage preferences. J Comp Biol. 2009; 16: 1539–1547. https://doi.org/10.1089/cmb.2009.0046 PMID: 19958082

64. Puigbó P, Bravo IG, Garcia-Valle S. CAcal: a combined set of tools to assess codon usage adaptation. Biol Direct. 2008; 3: 38. https://doi.org/10.1186/1745-6150-3-38 PMID: 18796141

65. Krogh A, Larsson B, von Heijne G, Sonnhammer EL. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol. 2001; 305: 567–580. https://doi.org/10.1006/jmbi.2000.4315 PMID: 11152613

66. Mulder N, Apweiler R. InterPro and InterProScan: Tools for protein sequence classification and comparison. Meth Mol Biol. 2007; 396: 59–70. https://doi.org/10.1007/978-1-59745-515-2_5 PMID: 18025686

67. Boratyn GM, Thierry-Mieg J, Thierry-Mieg D, Busby B, Madden TL. Magic-BLAST, an accurate DNA and RNA-seq aligner for long and short reads. bioRxiv 2018; https://doi.org/10.1101/390013

68. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol. 2013; 30: 772–780. https://doi.org/10.1093/molbev/msr060 PMID: 23329690

69. Castresana J. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol. 2000; 17: 540–552. https://doi.org/10.1093/oxfordjournals.molbev.a026334 PMID: 10742046

70. Lefort V, Longueville J-E, Gascuel O. SMS: Smart Model Selection in PhyML. Mol Biol Evol. 2017; 34: 2422–2424. https://doi.org/10.1093/molbev/msx149 PMID: 28472384

71. Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermiin LS. ModelFinder: fast model selection for accurate phylogenetic estimates. Nat Meth. 2017; 14: 587–589. https://doi.org/10.1038/nmeth.4285 PMID: 28481363

72. Guindon S, Delsuc F, Dufayard J-F, Gascuel O. Estimating Maximum Likelihood Phylogenies with PhyML. Meth Mol Biol. 2009; 537: 113–137. https://doi.org/10.1007/978-1-59745-251-9_6 PMID: 19378142

73. Nguyen L-T, Schmidt HA, von Haeseler A, Minh BQ. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol Biol Evol. 2015; 32: 268–274. https://doi.org/10.1093/molbev/msv003 PMID: 25371430

74. Pacheco MA, Cepeda AS, Bernotienė R, Lotta IA, Matta NE, Valkiūnas G, et al. Primers targeting mitochondrial genes of avian haemosporidians: PCR detection and differential DNA amplification of parasites belonging to different genera. Int J Parasitol. 2018; 48: 657–670. https://doi.org/10.1016/j.ijpara.2018.02.003 PMID: 29625126