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Mosquito-borne epornitic flaviviruses: an update and review

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Abstract

West Nile Virus, Usutu virus, Bagaza virus, Israel turkey encephalitis virus and Tembusu virus currently constitute the five flaviviruses transmitted by mosquito bites with a marked pathogenicity for birds. They have been identified as the causative agents of severe neurological symptoms, drop in egg production and/or mortalities among avian hosts. They have also recently shown an expansion of their geographic distribution and/or a rise in cases of human infection. This paper is the first up-to-date review of the pathology of these flaviviruses in birds, with a special emphasis on the difference in susceptibility among avian species, in order to understand the specificity of the host spectrum of each of these viruses. Furthermore, given the lack of a clear prophylactic approach against these viruses in birds, a meta-analysis of vaccination trials conducted to date on these animals is given to constitute a solid platform from which designing future studies.

INTRODUCTION

West Nile virus (WNV), Usutu virus (USUV), Tembusu virus (TMUV), Bagaza virus (BAGV) and Israel turkey meningo-encephalitis virus (ITV) are positive-sense, single-stranded RNA viruses, included in the mosquito-borne cluster of the genus *Flavivirus*, family *Flaviviridae* [1]. Their natural life cycle mainly involves birds and mosquitoes, whereas humans and other vertebrates are considered incidental hosts [2–5]. A remarkable hallmark of these arboviruses is their ability to invade new territories. The most recent examples of this feature are the introduction into Europe of USUV in 1996 [6], WNV lineage 2 in 2004 [7], BAGV in 2010 [8] and of TMUV into China in 2010 [9]. In avian hosts, these flaviviruses are considered as epornitic (capable of causing epizootics in birds). Consequently, we will refer to them in this review as mosquito-borne epornitic flaviviruses (MBEF). MBEF have been detected in an increasing number of bird species and can be deadly for a wide range of them. Moreover, when poultry flocks become infected by ITV and TMUV, high mortality, drop in egg production and heavy control measures constitute an economic burden for the infected countries.

Beside their impact on bird health and the poultry industry, MBEF are capable of infecting humans [10–13], except ITV, of which the zoonotic potential is still to be determined. Most human infections remain asymptomatic, but symptoms ranging from transient flu-like syndrome (fever, headache) to severe neurological illness and death can be observed in some cases of WNV and USUV infections [13, 14].

In this article, we will review the genome structure, classification, eco-epidemiology, pathology and preventive measures related to MBEF. We will list avian species currently known to be susceptible to the infection and we will provide an overview of vaccination trials conducted to date on birds to boost their immune system against these viruses.

Genome structure

The MBEF group are positive-sense, single stranded RNA viruses [15]. Spherical and enveloped virions measure 40–60 nm in diameter [1]. Their ~11 kb viral RNA genome contains a unique open reading frame (ORF) flanked by a capped 5′-terminal non-coding region (NCR) and a 3′-terminal NCR (Fig. 1). The two NCRs form specific secondary structures necessary for genome replication and translation, and are implicated in the pathogenicity of flaviviruses [16]. The single polyprotein encoded by the ORF is processed by viral and host proteases into three structural and seven non-structural proteins [1]. The structural proteins comprise: (1) an envelope protein E, involved in receptor binding, viral entry and

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Abbreviations: AMCR, American crow; BAGV, Bagaza virus; CNS, central nervous system; C protein, capsid protein; E protein, envelope protein; HOSP, house sparrow; IFN, interferon; ITV, Israel Turkey meningo-encephalitis virus; MBEF, mosquito-borne epornitic flaviviruses; M protein, membrane protein; NCR, non-coding region; NK, natural killer; NS, non-structural; ORF, open reading frame; PAMP, pathogen-associated molecular patterns; prM, membrane precursor; SPF, specific-pathogen-free; TE, truncated envelope protein; TMUV, tembusu virus; USUV, usutu virus; WNV, West Nile virus.

Supplementary material is available with the online version of this article.

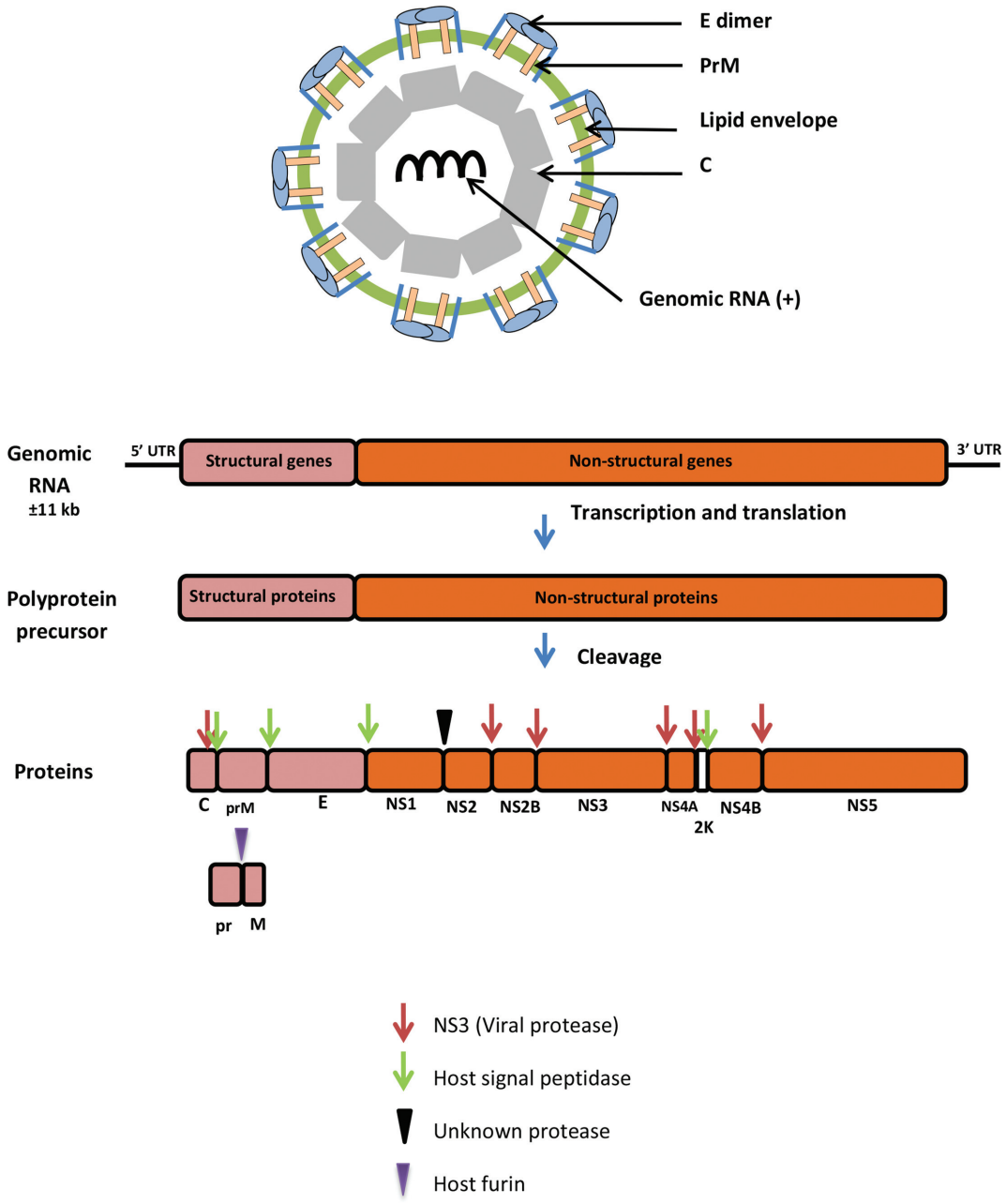

Fig. 1. Virion structure and genomic organization of epornitic mosquito-borne flaviviruses. The single-stranded, positive-sense RNA genome contains a single unique ORF, encoding for a polyprotein which is processed into three structural proteins (C, PrM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). UTR, untranslated transcribed region.

membrane fusion; (2) a membrane protein M, which results from the cleavage of a membrane precursor prM upon maturation of the virion; and (3) a capsid protein C, involved in the assembly of the nucleocapsid and its incorporation into new virions [17]. The E protein carries both flavivirus cross-reactive and virus-specific epitopes, and hence it constitutes the main target of neutralizing antibodies and the base of several vaccine candidates against these viruses [18]. Alternatively, a truncated E (TE) protein without a membrane anchor region can be used to increase secretion of the E protein ecotodomain, carrying major immunogenic epitopes [18]. The prM protein protects the E protein from premature fusion during the exocytosis of viral particles and participates in the folding and assembly of viral particles [1]. The prM-E proteins of flaviviruses can self-assemble into subviral particles, which share features similar to the antigenic structure of the virions [17]. Therefore, many vaccine candidates for the immunization of birds have been based on prM and E proteins.

The non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) regulate RNA transcription and replication [1], determine virus evasion mechanisms from the host immune system (e.g. limit interferon (IFN) gene expression by attenuating the signalling through the JAK/STAT pathway) [19, 20] and play an important role in avian host competence and virulence [21, 22]. Among these proteins, NS3 is a serine protease that cleaves NS2A/B, NS2B/NS3, NS3/NS4A and NS4B/NS5 [20]. This protein also has RNA helicase activity, allowing the genome to be unwound during viral replication, and RNA triphosphatase activity, involved in the dephosphorylation of the 5′ end of the genome before the addition of a cap [1]. NS5 is a highly conserved protein among flaviviruses and is also a multifunctional protein: at the N-terminus, it has methyltransferase activity required for the formation of mRNA (RNA capping); and at the C-terminus, it has an RNA-dependent–RNA-polymerase activity necessary for copying genomic RNA [1].

Lineages and strains

The MBEF members belong to the genus *Flavivirus*, family *Flaviviridae* [1]. This family is divided, according to the transmission routes of its members, into three clusters (Fig. 2): (1) arthropod-borne viruses, transmitted horizontally by mosquito or tick bites to vertebrate hosts and thus considered as dual-host viruses; (2) unknown vector flaviviruses, also called vertebrate-specific flaviviruses, presumed to infect only rodents or bats; and (3) insect-specific or mosquito-only flaviviruses that can replicate only in insects, especially mosquitoes [23].

The most important flaviviruses in regard to humans and animals belong to the first cluster, for which birds can act as the reservoir [23]. Among these, some are transmitted by ticks, mostly *Ixodes* sp. [24], and can severely impact the health of human (e.g. Tick-borne encephalitis virus) [25] or avian hosts, such as Louping-ill virus, which is deadly for the red grouse (*Lagopus lagopus*) [26].

Other arthropod-borne flaviviruses are transmitted by mosquitoes, with some being non-pathogenic for birds but highly virulent in humans, such as Murray Valley encephalitis virus [27] and Saint Louis encephalitis virus [28]. WNV, USUV, TMUV, BAGV and TMEV are the only mosquito-borne viruses having a known pathogenicity for birds (Table 1).

The MBEF members are serologically classified within two different groups: (1) the Japanese encephalitis serocomplex, including WNV and USUV, and (2) the Ntaya serocomplex, including AMEV and TMUV [29, 30] (Table 1).

Viruses from the Japanese encephalitis serocomplex: USUV and WNV

Isolates of USUV are currently classified into eight lineages (Africa 1, 2 and 3 and Europe 1, 2, 3, 4 and 5) [31]. Molecular studies on nucleotide and amino acid sequences of these isolates from vectors, birds and humans reveal significant substitutions, some of which have been suggested as being related to viral neuro-invasiveness [32]. The effective role of such candidate mutations in the development of both viral infectivity and virulence remains to be determined.

At present, up to nine lineages have been proposed to classify WNV strains [33]. Lineage 1 is subdivided into clades 1a and 1b (or Kunjin virus) and 1 c [34], and is the most widespread in USA (NY99 strain), Africa (KN3829), Europe and the Middle East [33]. Virulence is highly variable among WNV lineages. For instance, lineage 3 (Rabensburg virus) has never been isolated from humans and did not experimentally infect mammalian or avian cell cultures, the house sparrow (*Passer domesticus*) (HOSPs) or specific-pathogen-free (SPF) chicken eggs [35]. On the contrary, WNV lineages 1 and 2 have been responsible for major outbreaks in animals and humans [35, 36]. Viral strains from the same lineage (and clade) can also express variation in pathogenicity. For instance, despite the high genetic relatedness between strains KN3829 and NY99 (a total of 11 amino acid differences between the strains) [22], the latter exhibits a strikingly different avian virulence phenotype, eliciting significantly higher viraemia and mortality in the American crow (*Corvus brachyrhyncos*; AMCR) [22, 37]. A mutation in the NS3 gene resulting in a T249P amino acid substitution was involved in increased pathogenicity in AMCR [38], and this mutation was proposed as a key determinant of WNV pathogenicity. Furthermore, the NS3-249 residue was shown to be under strong positive selective pressure because birds can drive adaptive evolution in WNV [38]. However, the mere presence of Pro at NS3-249 was neither sufficient nor necessary to enhance the virulence of WNV strains in theHOSP [39, 40], red-legged partridge [41] and SPF chicken [42]. Variation in virulence for avian species in regard to this mutation remains unexplained. Nonetheless, one study showed that WNV virulence in AMCR is correlated with increased ATP hydrolysis due to direct interaction between the NS3-249 residue and unknown host factors [43]. Helicase activity, however, did not differ between NS3 proteins with a proline or threonine at position 249, and thus could not explain the *in vivo* effects in AMCR [43]. Other studies showed that the NS3-249 residue modulates replication in avian leukocytes [22, 44] and hence could affect the host immune response in a temperature-dependent manner and under the control of NS proteins [22].

Viruses from the Ntaya serocomplex: BAGV, ITV and TMUV

The BAGV strains comprise isolates from the Central African Republic, India and Spain, with high nucleotide identity (>92 %) [8]. ITV includes strains from Israel and South Africa, with <0.9 % of divergence [30]. Both viruses have shown cross-neutralization activity and nucleotide sequence identity >84 % and were proposed to be considered as a single virus species, named avian meningoencephalitis virus [23, 30]. However, the International Committee on Taxonomy of Viruses species

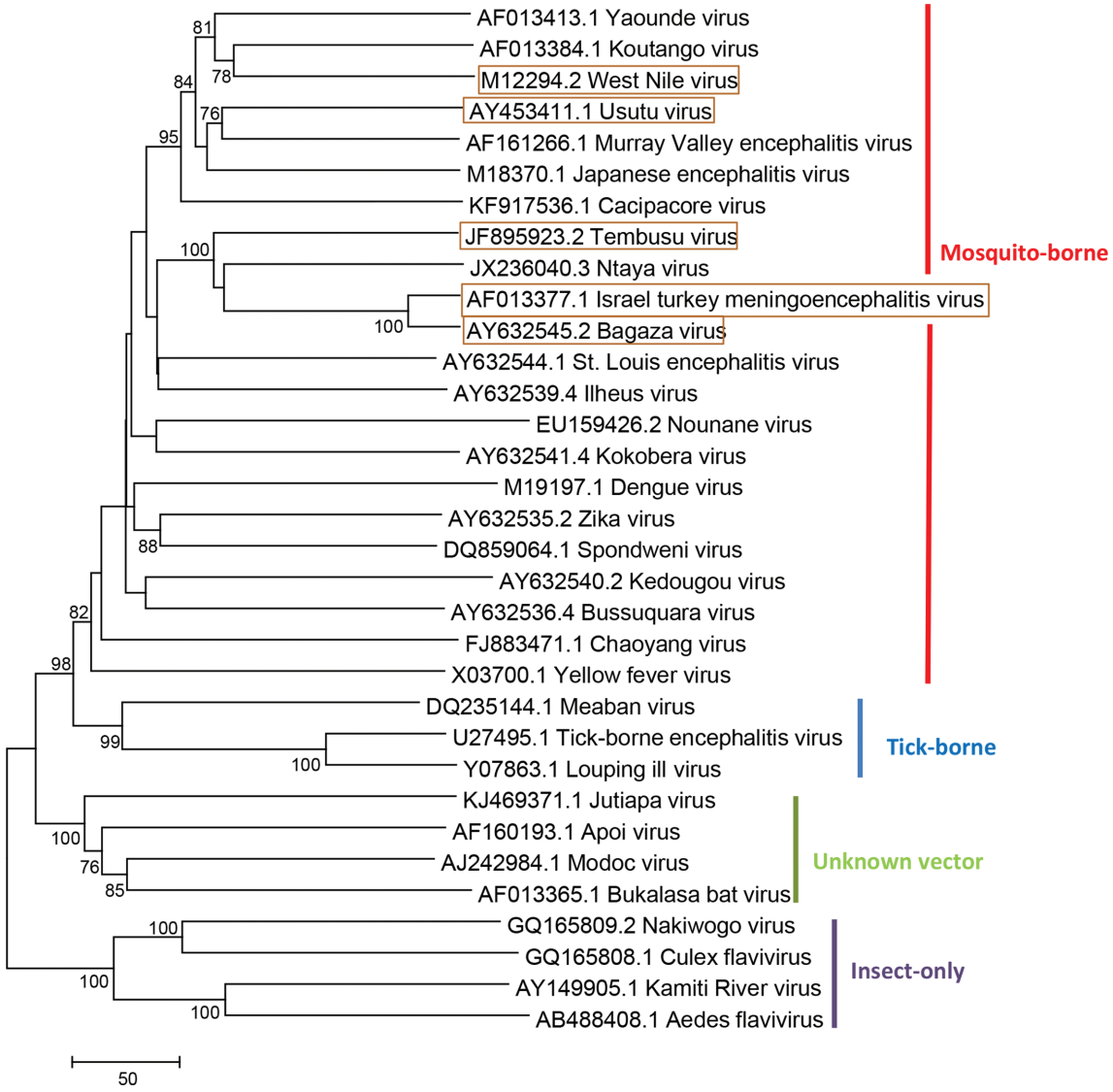

Fig. 2. Phylogeny of conserved partial gene sequences coding for the non-structural protein 5 of certain representative strains from the family *Flaviviridae*. ClustalW (implemented in Geneious 10.2.3) was used to create multiple alignment for the sequences. The phylogenetic tree was constructed from the sequence alignment by the maximum likelihood method based on the Kimura 2-parameter model [149] with a gamma distribution (five categories) and invariant sites (G+I) computed with MEGA 7 [150]. The tree is drawn to scale, with branch lengths measured according to the number of substitutions per site. Data were bootstrap re-sampled 500 times; values ≥70 % are shown next to the branches. Mosquito-borne epornitic flaviviruses are framed.

demarcation criteria for viruses of the genus *Flavivirus* include geographic, vector, host and disease associations and ecological characteristics [45] and, thus, these viruses should still be considered as separate species [15] because they differ in some of these aspects (Table 1). TMUV is a genetically distinct member of the Ntaya virus group and includes highly homologous isolates that were previously considered separate virus species, including Sitiwan virus [46], duck egg-drop syndrome virus [47], Perak virus [48] and Baiyangdian virus [49]. Genetic features underlying the infection and disease outcome associated with these viruses are still poorly understood. Recently, N-glycosylation on residue 154 of TMUV E protein has appeared as a determinant of pathogenicity in ducks, as shown for WNV in other avian species [50–53]. In fact, an S156P mutation in the E protein of one TMUV strain (FX2010) resulted in loss of the E-glycosylation motif, leading to limited virus replication and the abrogation of vector-free transmission of TMUV in ducks [54].

Table 1. Epornitic mosquito-borne flaviviruses: classification and main epidemiological and pathological features

MBEF	Serocomplex	First detection in birds	Most susceptible bird species	Major clinical signs	Lesions	Geographic distribution
WNV	Japanese encephalitis	1953 in Egypt. WNV lineage-2 : 2004 in Europe 1999 in North America	Order: *Passeriformes* (*Corvidea*)	Sudden death Neurological signs	Encephalitis Necrosis in liver, heart and spleen	Worldwide North America: frequent Europe: occasional epizootics Elsewhere: infrequent
USUV		1972 in Africa 1996 in Europe	Orders: *Strigiformes* *Passeriformes* (*Turdus merula*)			Africa: sporadic Europe: seasonal epizootics
BAGV	Ntaya	2010 in Spain	Phasianids: partridge		Encephalitis Necrosis in liver, heart and spleen Oophoritis	Spain: sporadic Central African Republic, Cameroon, Mauritania, Senegal, India: reported in mosquitoes
ITV		1958 in Israel	Phasianids: turkey	Sudden death Neurological signs		Israel: sporadic South Africa: sporadic
TMUV		1976 in Malaysia	Duck, goose	Egg drop		Southeast Asia (Malaysia, Thailand, Indonesia, China) Enzootic in China and Malaysia

Geographic repartition

USUV

USUV was detected for the first time in 1959, by B.R. McIntoch, from *Culex neavi* (historically named *Culex univitatus*) captured near the Usutu river in Swaziland, South Africa [55].The virus was later detected in mosquitoes in several African countries, until its identification as the causative agent of mass mortality in the Eurasian blackbird (*Turdus merula*), barn swallow (*Hirundo rustica*) and great grey owl (*Strix nebulosa*) in and around Vienna (Austria) in 2001 [56]. Proof of the introduction of this virus in Europe prompted a retrospective analysis of tissue samples, collected from dead blackbird in the Tuscany region of Italy in 1996 [6]. The results were positive for USUV, providing evidence of its circulation before its isolation in dead birds in Austria. In subsequent years, the virus range expanded to several European countries and it was detected in avian species (Appendix 1, available in the online version). Senegal has been suggested as the origin for virus introduction in Central Europe [57], and the identification of an African strain in August 2015 from the carcases of two juvenile great grey owls in Berlin (Germany) has revealed continuous introduction of the virus [58].

WNV

This virus has disseminated globally since it was first isolated in the West Nile province of Uganda in 1937 [59], and has had a major impact on human, equine and avian health [36]. The virus was first isolated in avian species in Egypt in 1953 from the blood of two rock pigeons (*Columba livia*) and one hooded crow (*Corvus cornix*) [60]. It has since been associated with two major epornitics, the first in the migratory white stork (*Ciconia ciconia*) and domesticated goose (*Anser anser domesticus*) in Israel, between 1997 and 2000 [61], and the second in AMCR in the USA, where strain NY99 was introduced in 1999 [62]. High mortality in birds has been a common feature of WNV activity in the USA, with infection detected in dead birds of to up to 342 species [63]. Besides, the virus has resulted in infection since its emergence in over 27 000 horses [64] and in neuro-invasive disease in 48 183 humans (2163 deaths), according to the Centers for Disease Control and Prevention [65]. In contrast, WNV only sporadically caused infections and neurological illnesses in humans and horses in Europe [36]. Wild bird mortality events have been even more infrequent, with small and isolated episodes and a limited number of avian species testing positive for WNV infection (24 species to date, as shown in Appendix 2). This variability in the clinical impact of WNV infections in humans, horses and birds has been linked to both intrinsic (e.g. vector competence, mosquito feeding preferences and longevity, and host immunity) and extrinsic factors (e.g. host and mosquito density, composition of host and vector populations and environmental conditions) [59, 66].

BAGV and ITV

Bagaza virus was first isolated in the Bagaza district of the Central African Republic (CAR) in 1966, from a pool of *Culex* spp. mosquitoes [67]. Subsequently, this virus has been isolated from various species of mosquito in Central and West African countries [68], and in India, where serological investigations implicated its involvement in human encephalitis [10]. In September 2010, BAGV was found to be associated with high mortality in game partridge and pheasant in southern Spain [8, 69]. This was the first time the virus had been detected in Europe and the first proof of BAGV adaptation to avian species. The closely related ITV

has been reported as affecting turkey (*Meleagris gallipavo*) since 1958, in Israel and in South Africa [70]. Apart from Israel, ITV has been reported only in South Africa, but also in the domesticated turkey [71].

TMUV

This virus was first detected in mosquitoes in Kuala Lumpur in 1955 [46], and it has frequently been isolated from *Culex* and *Aedes* mosquitoes in Malaysia [72] and Thailand [2]. Sitiawan virus was the first TMUV strain reported to cause encephalitis and retarded growth in broiler chickens in Malaysia [46]. In 2010, egg-drop syndrome and encephalitis were observed in both meat and laying ducks in China, and TMUV was identified as the causative agent [73]. In addition, a similar TMUV disease also emerged in duck flocks in Malaysia in 2012 [48] and in Thailand in 2013–2014 [74]. TMUV has not been associated with human disease, but detection of neutralizing antibodies to the virus has been reported in human sera from Malaysia and Indonesia [75]. Detection of antibodies against TMUV in healthy duck industry workers in Shandong, China provided evidence of TMUV duck-to-human transmission [12]. Although it has not been shown, to date, to result in either clinical manifestations or viraemia in non-human primates [76], the potential emergence of strains virulent for humans should be considered [12].

Life cycle and host range

Viruses in the MBEF group are maintained in nature by a cycle (Fig. 3) involving adult ornithophilic mosquitoes, principally *Culex* spp., as vectors, and competent birds (those that express sufficiently high viraemia levels to infect naive mosquitoes) as the reservoir [2, 4, 5, 13, 77]. BAGV,

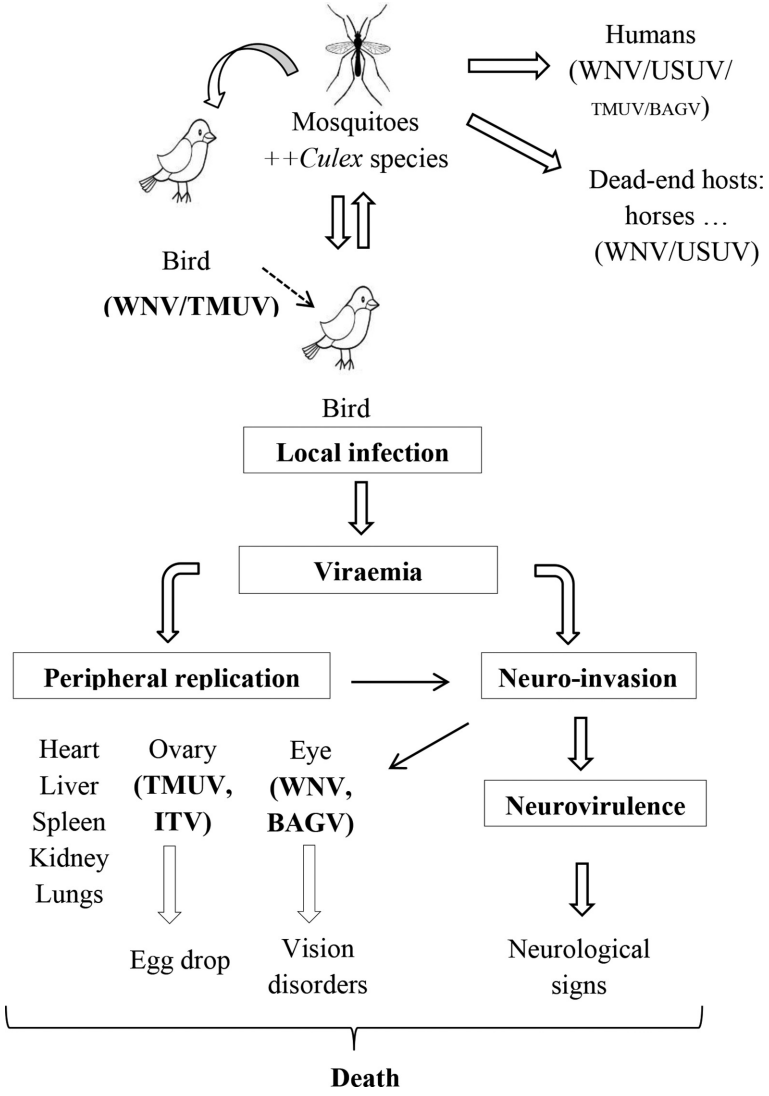

Fig. 3. Basic transmission cycle and pathogenesis of mosquito-borne epornitic flaviviruses.

WNV, USUV and TMUV can incidentally infect many hosts, including humans [10–13], with varying degrees of pathogenicity, ranging from asymptomatic infection to severe neurological illness – attributed to WNV [14] and, less frequently, to USUV [13]. While little is known about other potential hosts of BAGAV, ITV and TMUV, both WNV and USUV have been shown to naturally infect dog, bat [78], red deer [5, 79] and equids [80]. Only in equids have encephalitis and death following WNV infection been reported [64]. The vertebrate host range of WNV even encompasses other animals such as reptiles (e.g. alligator, snake) and amphibians (e.g. frog), yet only a small number of host species contribute to vector-borne transmission [5]. Some tick species can replicate WNV, but their role in the introduction and maintenance of WNV infections remains uncertain [81, 82].

Migratory birds are thought to be the principal agent for the global spread of WNV and the introduction of USUV to Europe. Avian migratory status did not appear to reduce WNV viraemia titres or inhibit the migratory behavior of passerines, demonstrating that long-distance migratory birds can carry the virus to new territory [83, 84]. In addition, infectious viraemia was detected in birds during autumn migration in the Atlantic and Mississippi flyways in 2002 and 2003 [83]. Isolation of WNV and detection of virus activity by RT-PCR in the brain of white stork in Israel, during migration from Europe within two days of arrival at a stop-over site, provides further evidence of virus dispersal via these hosts [61]. A dispersal pattern of WNV across the USA via avian flyways was phylogenetically predicted [85]. Similarly, long-distance migratory birds were suggested as playing a key role in the introduction of USUV in Europe, because the genetic structure of the virus follows the geographical location and pattern of migratory flyways [57].

The MBEF group has a heterogeneous spectrum of pathogenicity according to avian species. Since its emergence in Europe, evidence of USUV circulation has been detected in at least 93 species from 35 families (Appendix 1). Some of these species showed evidence of silent infection, which was revealed by anti-USUV antibodies. However, the presence of viral RNA in dead birds of 36 species, mainly from the orders Passeriformes and Strigiformes, may indicate a specific virulence of the virus towards these avian species (Appendix 1). Eurasian blackbird (*Turdus merula*) is the species most affected in Europe (Appendix 1). In Germany, USUV has been demonstrated as causing a 15.7 % decline in the population of *T. merula* during the five years following its first detection in the southwest of that country in 2011 [86]. As a general rule, *Passeriformes* (especially *Corvidae*) and *Charadriiformes* (*Laridae*) are considered highly susceptible to WNV infection, with differences in viraemia levels depending on the species and viral strain [70]. The emergence of BAGV in Spain in 2010 resulted in high mortality rates in two game bird species, red-legged partridge (*Alectoris rufa*) and common pheasant (*Phasianus colchicus*) [8] (Appendix 3). Following experimental infection with BAGV, red-legged partridges showed a mortality rate of 30 % [87], while grey partridges (*Perdix perdix*) showed 40% of mortality with severe neurological symptoms, but the level of viraemia was not sufficiently high in the latter species for it to be considered a competent host, in contrast to the former [88].

Fatal disease has been reported in turkeys infected with ITV [89] (Appendix 4), while TMUV has frequently been reported in ducks and occasionally in chickens and geese [46, 90] (Appendix 5).

The age of birds also seems to be an important factor in determining the course of mosquito-borne viral infections. Increased duration or intensity of viraemia in nestlings and juveniles, compared to adult birds, was noted after infection with different lineages of WNV [70]. Young ducks and turkeys are more susceptible to infection by TMUV and ITV, respectively, as they show more severe symptoms and lesions along with a lower neutralizing antibody response and a higher mortality rate [71, 91, 92]. There are no studies to date addressing the effect of age in regard to susceptibility to USUV and BAGV infections.

Beside age, there is an influence of gender on the morbidity and severity of ITV- and BAGV-associated diseases, with the female being more susceptible than the male in turkey [71], partridge [87] and pheasant [93].

Non-vector-borne transmission

The capability of MBEF to be transmitted in a vector-borne free manner is variable.

USUV

Contact transmission of USUV did was not possible in laboratory experiments in chicken (*Gallus domesticus*) [94] and domestic goose [95], species in which lethal infection has not been described to date. The use of susceptible bird species, including Passerines or Strigiformes, might be more useful in investigating the occurrence of direct USUV transmission.

WNV

In humans, cases of transmission of WNV through blood transfusion, organ transplantation, intra-uterine exposure and breastfeeding have been reported [11]. In avian hosts, contact transmission of WNV has been demonstrated in six bird species: common goose [96], chicken [97], ring-billed gull (*Larus delawarensis*), blue jay (*Cyanociatta cristata*), black-billed magpie (*Pica hudsonia*) and AMCR [98]. WNV-contaminated water infected the common grackle (*Quiscalus quiscula*), HOSP and AMCR [98]. Besides, oral transmission was experimentally demonstrated after ingestion of WNV-infected mice by five bird species: great horned owl (*Bubo virginianus)* [98, 99], eastern screech owl (*Megascops asio*) [100], black-billed magpie (*Pica hudsonia*), AMCR [98] and American kestrel (*Falco sparverius*) [99]. An AMCR showed viraemia after ingestion of an infected HOSP carcass, and the same was observed in a house finch

after consumption of an infected mosquito [98]. This observation supports the hypothesis that WNV-infected birds in nature, especially corvids, constitute a source of contamination for birds of prey via the oral route [101].

BAGV and ITV

Direct transmission of BAGV in experimentally infected partridge remains controversial. While some researchers have demonstrated direct transmission in red-legged partridge [87], a recent study confirmed the absence of this transmission path in grey partridge [88]. Interestingly, the presence and persistence of viral load in feather pulp was found in Gyr–Saker hybrid falcon (*Falco rusticolus* × *Falco cherrug*) infected with WNV [102], in red-legged partridge [87] and in grey partridge [88] infected with BAGV, suggesting possible transmission via feather-picking. Furthermore, ITV was detected and amplified from feather pulp and this technique was proposed to evaluate the proper administration of live vaccines [103]. However, contagion did not occur in turkey experimentally infected with ITV [104]. Similarly, vertical passage of this virus was not found using the turkey as experimental models [71].

TMUV

TMUV is considered a contagious virus since horizontal transmission through direct contact, ingestion or inhalation of contaminated materials in duck (*Anas platyrhynchos)* and goose was demonstrated under both field and laboratory conditions [9, 91, 105–107]. Besides, vertical transmission was demonstrated in TMUV-infected duck [108]. The transmissibility of TMUV in duck is largely attributable to the E protein. Recently, the I domain of E protein has been found to directly impact virus replication in duck lung, thereby modulating virus shedding which is crucial for vector-free transmissibility of TMUVs in duck [54]. Besides, the amino acid Ser at position 156 in the E protein was shown to be responsible for virus tropism and transmission in duck, because a mutation of this residue led to the loss of N-linked glycosylation and the abrogation of non-vector-borne transmission of TMUV in duck [54].

Pathogenesis and immune response

The pathogenesis of MBEF proceeds in three major phases: (1) local infection and primary viraemia, (2) virus spread and peripheral replication and (3) neuroinvasion (infection of the central nervous system (CNS) and neurovirulence (damage to neuronal cells) [109] (Fig. 3).

After experimental inoculation, primary viraemia usually develops in less than 24 h [91, 104, 110, 111]. A viraemia level of 10^5p.f.u. ml^{-1} is necessary to infect mosquitoes with WNV after a blood meal [112]. The dose and number of feeding mosquitoes directly affects the speed at which WNV spreads systemically [113]. Development of the disease results from the invasion of major organs such as the liver, spleen, kidney, heart and CNS, in which the virus induces autophagy, apoptosis and the production of cytokines and chemokines, which promote leukocyte invasion, inflammation and necrosis [1]. Typical neurological signs appear at this stage, such as ataxia and paralysis [48, 87, 93, 114, 115] and non-specific signs, such as lethargy, ruffled feathers and weight loss [8, 69, 90, 91, 116]. Lesions are likewise developed and include necrotizing hepatitis, splenitis, myocardial degeneration and/or myocarditis, necrosis of striated muscles, non-suppurative encephalitis and neuronal necrosis [29, 48, 69, 74, 108, 110]. Haematogenous and/or neuronal dissemination of WNV and BAGV to the eye has been described in birds showing blindness [117, 118]. Severe egg drop (up to 90 %) and mortality (up to 30 %) in laying turkeys infected with ITV, and in layer chicken, ducks and geese infected with TMUV, have been reported [49, 71, 73]. The corresponding lesions are oophoritis, ovarian atrophy, haemorrhage and necrosis [9, 49, 71]. Although egg production can recover within 3–4 weeks after epizootic TMUV infection, both fertility and hatchability rates of eggs from breeding ducks were permanently lowered [119]. Reduced sperm production, spermatocyte swelling and vacuolar degeneration occurred in the testes of infected male ducks, with focal lymphocytic infiltration in the later stages [111]. In ducklings, TMUV infection caused hyperglycaemia (due to acute pancreatitis), neurological disease [47] and multi-organ failure leading to death [91].

In a manner similar to humans and horses, birds utilize the 2′−5′-oligoadenylate synthase pathway in the innate immune response against these flaviviruses [120]. This pathway ultimately induces apoptosis with other components of the innate immune response, including IFNs, inflammatory cytokines, complement factors, natural killer cells (NK) and autophagy to inhibit viral replication [1, 76]. Neutralizing antibodies, which primarily target the viral E glycoprotein, and antibodies against NS proteins constitute the major humoral immune response to flavivirus infection [121]. Seroconversion, as well as persistence of antibodies, is variable among birds. Importantly, maternal antibodies in young chicks can serve for rapid protection from WNV and TMUV infections [120, 122]. In addition to effective host humoral immunity, cellular immunity is triggered to control viral infection and dissemination [1]. Flaviviruses have developed numerous strategies to avoid the host immune system, including the limitation of initial steps of PAMP detection, type I IFN signalling by blocking the host gene expression and inhibition of the complement system and NK cells [19].

Once infected with WNV, most susceptible birds remain asymptomatic because the immune response eliminates the virus from the organism within two or three weeks [98]. In some cases, infection with WNV can become persistent and viral RNA may be detected for several months after infection, as has been demonstrated for house finch (*Haemorhous mexicanus*), HOSP, western scrub-jay (*Aphelocoma californica*), kea (*Nestor notabilis*) and rock pigeon (*Columba livia*) [123–125]. However, the question of whether

persistently infected birds could trigger a mosquito–bird transmission cycle remains unresolved [123].

Prevention and control

To monitor MBEF circulation, several approaches have been used in many European countries, including sero-surveillance in birds and viral identification in dead birds and in pooled mosquito samples [126, 127].

Given the lack of specific treatment for MBEF infection in birds and mammals, preventive measures should be applied to decrease the risk of infection. Mosquito control and indoor housing of captive animals is suggested to prevent mosquito bites [128]. The use of pyrethrinoid-based insecticides and the elimination of mosquito habitats where these insects can lay eggs should be implemented in affected areas [69]. Widespread ultra-low-volume application of insecticides has been successfully applied to reduce human WNV infection [129, 130], but this alternative is challenging in wild territories in regard to free-ranging birds. Lowering viraemia in competent avian hosts is another solution to prevent infection following mosquito bite [131] and, thus, to prevent human infections with the two major MBEF members, WNV and USUV. Biosecurity measures and the development of vaccines are crucial in preventing major economic losses in the poultry industry due to ITV and TMUV infections. While no vaccine against USUV or BAGAV has been tested on birds to date, many others have been developed against WNV, ITV and TMUV and tested in these animals.

Vaccines against WNV

Inactivated vaccines

The first licensed WNV vaccine for veterinary use was dedicated to the horse. A formalin-inactivated WNV lineage 1 vaccine was developed in 2003 by Fort Dodge Animal Health and commercialized in the USA under the trade name West Nile-Innovator (in Europe: Equip WNV Zoetis, previously Duvaxyn WNV). This vaccine elicited variable antibody responses across bird species and the majority of vaccine trials were not conclusive, as they lacked a virus challenge test (Appendix 6). A three-injection scheme with this vaccine was, however, suggested for falcons as it was able to provide protection from lethal testing, although minor clinical signs and lesions, as well as viraemia and virus shedding, occurred following the vaccination/challenge test [132].

Subunit/DNA vaccines

Subunit vaccines based on WNV TE proteins were trialled in domestic goose, red-legged partridge and Hawaiian goose ēnē (*Branta sandvicensis*), but protection was assessed only in partridge, which remained fully protected after a challenge test (Appendix 7A).

Two DNA vaccines encoding the TE protein of WNV lineages 1 and 2 without prM caused local inflammation at the site of injection and did not prevent death in all vaccinated falcons after lethal testing [133]. DNA vaccines expressing WNV prM and E proteins were trialledin birds, including the pCBWN vaccine and the Fort Dodge WN-Innovator DNA equine vaccine (Overland Park, KS) (Appendix 7B). The former was shown to fully protect fish crow (*Corvus ossifragus*) via the intramuscular route [134]. In contrast, the latter failed to induce antibody response in island scrub-jays (*Aphelocoma insularis*) [135] and did not prevent mortality, lesions and high viraemia levels after a challenge test in western scrub-jay (*Aphelocoma californica*) [133]. For large-scale immunization, oral administration of pCBWN was trialled in AMCR [136] and fish crow [134] but failed to provide protection in either species.

Chimeric vaccines

Using live attenuated strains of other viruses as a genetic backbone, multiple versions of chimeric vaccines against WNV have been designed and explored for immunogenicity in birds (Appendix 7C). A recombinant live canarypox ALVAC viral vector expressing WNV prM and E proteins, RecombiTEK, Merial-Sanofi Aventis, was licensed in 2004 for veterinary use [137]. Vaccine safety was not satisfactory as the vaccine induced local inflammatory and necrotic lesions at the injection site. Besides, it failed to induce an immune response in western scrub-jay [138]. However, three injections succeeded in reducing mortality after virus challenge in falcon [132]. A recombinant adenovirus vaccine, expressing WNV E or NS3 proteins, induced a specific antibody response in Japanese quail (*Coturnix japonica*) but the protection level was not assessed [139].

Three chimeric vaccine candidates, currently under trial for human, use were tested in birds. The first was ChimeriVax-WN, where WNV prM and E protein-coding genes were incorporated into the genome of the 17D non-structural genes of yellow fever virus. In the second, chimeric WN/DEN4, prM and E protein-coding genes of dengue virus type 4 were replaced with the corresponding genes from WNV while in the third, WN/DEN4-3'Δ30, a 30-nucleotide deletion in the non-coding region of the DEN4 component of chimeric WN/DEN4 was introduced. These vaccines failed to prevent clinical symptoms, viraemia or death after the challenge test as they could not be replicated in these avian hosts, probably due to the fact that the backbone viruses were not adapted to these hosts [140, 141].

Heterologous vaccines

To assess the advantage of flavivirus cross-reactivity for heterologous protection, an attenuated vaccine against ITV was tried in goose, and resulted in 39–72 % protection against WNV challenge in field-vaccinated birds [142].

Vaccines against ITV and TMUV

Since the emergence of ITV in Israel, commercial attenuated virus vaccines (Biovac Biological Laboratories, Akiva, Israel and Phibro, Beth Shemesh, Israel) based on virus strain JQ4E4 [143, 144] have been used in that country as a routine control strategy for the disease. Minor clinical signs have often been observed after vaccination [143].

To date, attenuated and killed vaccines have been commercialized to protect ducklings and layer ducks against TMUV, including Duck Tembusu Virus Vaccine Live (FX2010-180P strain) (ZHENGYE, Jilin, China), attenuated by serial passage in chicken embryo fibroblasts [107], and an inactivated TMUV vaccine (HB strain, Rinpu, Tianjin, China) (Appendix 8).

Attenuated *Salmonella typhimurium* SL7207 (pVAX-C) has been used as a vehicle in oral delivery of TMUV prM and E antigens to ducks [18]. Alternatively, another study used this attenuated bacteria to immunize ducks with TMUV C protein to induce a systemic immune response [145]. These two vaccines showed 100 % survival among duck, with minor clinical signs after lethal testing [18, 145].

To develop multivalent vaccines, recombinant avian viruses, such as Duck enteritis virus and Newcastle disease viruses, were used as vectors for prM/E [146–148] and succeeded in fully protecting duck following a challenge test.

Conclusions

Birds play a key role in the life cycle of many flaviviruses as amplifying hosts, with an important contribution to their transmission and spread either locally or to new territories. MBEF are highly pathogenic for certain avian species. Furthermore, WNV and USUV occasionally cause severe neurological disease in humans and, thus, constitute a concern for both veterinary and public health.

Eradication of these pathogens is virtually impossible, because the viruses are maintained in a complex life cycle involving several animal reservoirs, some of which remain unknown. Preventive measures remain the only solution to help reduce and control their circulation, but such measures are hampered by the unresolved transmission routes of these viruses, the limited cost-effectiveness of vaccination and the underestimation of seasonal infection and mortality rates. In fact, MBEF infections often occur unnoticed, because many birds develop an asymptomatic form of the disease or die without collection by competent authorities. Formulating cheap and completely protective single-dose or oral vaccines would be the golden goal for simple and large-scale immunization of domestic and wild birds. More studies need to be carried out to evaluate the actual prevalence and incidence of these MBEF, to study their pathogenesis and to fully elucidate their life cycles and transmission routes, as preliminary steps towards the preservation of wild bird species, the reduction of the impact on domestic birds and the prevention of human infections.

Author bio
The author is currently a PhD student in the Morphology and Pathology Department of the Veterinary Faculty of Liège. Her primary research interest is the pathogenesis of USUV.

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