Review Article

Title:Potential Role of Birds in Japanese Encephalitis Virus Zoonotic Transmission and Genotype Shift

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Abstract: Japanese encephalitis (JE) is a vaccine preventable disease caused by the Japanese encephalitis virus (JEV), which is primarily prevalent in Asia. JEV is a Flavivirus, classified into a single serotype with five genetically distinct genotypes (I, II, III, IV, and V). JEV genotype III (GIII) had been the most dominant strain and caused numerous out breaks in the JEV endemic countries until 1990. However, recent data shows the emergence of genotype I (GI) as a dominant genotype and it is gradually displacing GIII. The exact mechanism of this genotype displacement is still unclear. The virus can replicate in mosquito vectors and vertebrate hosts to maintain its zoonotic life cycle; pigs and aquatic wading birds act as an amplifying/reservoir hosts, and humans and equines are the dead end hosts. The important role of pigs as an amplifying host for JEV is well known. However, the influence of other domestic animals especially birds that live in high abundance and close proximity to human is not well studied. Here, we strive to briefly highlight the role of birds in JEV zoonotic transmission, discovery of birds as a natural reservoirs and amplifying host for JEV, species of birds susceptible to JEV infection, and the proposed effect of JEV on poultry industry in future perspective which have been neglected for a long times. We also discussed the recent *in vitro* and *in vivo* studies which show that the newly emerged GI viruses replicated more efficiently

in bird-derived cells and ducklings/chicks than GIII, and an important role of birds in the JEV genotype shift from GIII to GI.

Keywords: Japanese encephalitis virus, birds, genotype shift, JEV genotype I, JEV genotype III

1. Introduction

JEV causes neurological disease which is one of the leading viral encephalitis in the world [1]. According to World Health organization (WHO) more than 24 countries from Asia and Western Pacific regions have exposed to JEV, where it accounts for ~35,000 to 50,000 cases and 10,000 to 15,000 deaths each year [2]. However, the exact numbers of cases are probably remains under reported [3].

The majority of human infections are asymptomatic, and many symptomatic cases result in meningitis, encephalitis or flaccid paralysis, and are fatal or cause devastating long-term neurological sequelae. JEV epidemics were originally reported from Japan in the nineteenth century, and the virus was first time isolated in 1935 from an infected human brain samples in Tokyo [4]. JEV infections occur across a large range of Asian countries with outbreaks occurring in Japan, China, Taiwan, Korea, the Philippines, and India [5]. JEV cases occurrence in Nepal, India, Papua Guinea, Pakistan, and Australia, suggesting that this virus is going to expand its geographic range in future [6, 7]. In 2017, a whole JEV genome was identified by unbiased RNA sequencing in a patient coinfected with yellow fever in Cunene province, Angola, raising the possibility that the geographic range of JEV might be greater than previously thought [8]. This shows that JE might be going to become a public health problem of intercontinental concern [9].

JEV has a positive sense RNA genome belonging to Flavivirus genus within *Flaviviridae* family, harbor three structural and seven non-structural proteins. JEV life cycle contained both invertebrates (mosquitoes) as well as vertebrates (wild birds and pigs). On the basis of phylogenetic investigations, JEV is classified

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into a single serotype with five genetically distinct genotypes (GI, GII, GIII, GIV, and GV). GIII had been the most prevalent strain with a number of epidemics in past. But, recent studies report the emergence of GI strain as a leading JEV genotype [10]. Since a few years, JEV isolates from Japan, Republic of Korea, and China were sorted out under JEV GI, although these regions were having GIII endemic history [10, 11]. In addition, the more divergent genotype V strains (amino acid divergence from 8.4% to 10.0% compared to genotypes I-IV) have been detected in Malaysia [12], Korea [13] and China [14], and may be covered poorly by existing genotype III-based vaccines. There is also concern that JEV could spread to Americas and Europe, much like the West Nile virus (WNV) did, as North American field-collected *Culex* mosquitoes and experimentally exposed *Culex* mosquitoes from Europe were found susceptible to JEV infection [15-17]. Furthermore, several avian species in North America are susceptible to JEV and can possibly serve as amplification hosts [18]. The spread of arboviruses such as WNV and JEV can occur by wind-blown mosquitoes, migrating viremic birds or anthropogenic activities [9, 19].

Pig's role as an amplification host for JEV has been well demonstrated in previous studies. Birds' role as an amplification/reservoir host has been poorly investigated. Recently, JEV epidemics are reported from areas with low pig population [20]. Additionally, the JEV genotype shift from GIII to GI has reported in some countries where pig-farming is not common, such as Malaysia [21], India [22], and Korea [20]. Human infection does not contribute to the JEV transmission and the human vaccine does not reduce transmission of JEV in the reservoir community, no herd immunity is generated, and vaccination has to be continued indefinitely. In this interactive review, we have highlighted the role of birds in JEV transmission, discussed the role of birds in JEV genotype shift from GI to GIII, and proposed effect of JEV on poultry industry in

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future perspective. Thus, we can contain JEV spread while taking possible countermeasures that can blunt their impact on public health as well as for veterinary concerns.

2. JEV zoonotic transmission

Mosquito-borne zoonosis includes JEV life cycle involved both invertebrates (mosquitoes) as well as vertebrates (wild birds and pigs). The JEV is transmitted by several *culicine*, *aedes*, *anopheles*, and *armigeres* mosquito species. *Culex* (*Cx*.) species mostly involved in transmission cycle of JEV. In initial investigations from Japan, relative abundance of each mosquito species caught in baited traps and their JEV infection status were compared when implicating vectors in transmission [23]. It was noticed that *Culex tritaeniorhyncus* is the primary vector for JEV transmission, which was strengthened later by laboratory experiments indicating this mosquito's competence for JEV replication and transmission [23, 24]. However, recent studies showing that other *Cx.* species are also competent vector for JEV [16, 17, 25].

In addition to mosquitoes as a vector, pigs and ardeid birds act as an amplifying/reservoir host [26]. Theoretical models of vector-borne pathogen transmission demonstrate that the pathogen transmission rate particularly depends upon the proportion of vector blood meals taken from competent hosts versus dead-end hosts [27]. Usually, JEV transmitted from infected pig/bird to non-infected by mosquitoes but recently its reported that this could be independent of the vector in pigs [28]. Pigs serve as amplifying hosts because they develop sufficient viral titers to support further infection of mosquitoes [29].

Previous studies reports dominance of pigs as amplifying hosts, but recently this concept has been challenged, as some countries such as Bangladesh with very little pig population also have appreciable burden of Japanese encephalitis in humans [30]. This reveals the presence of some other potential hosts that amplifying JEV. Recently, we have detected JEV in mosquitoes collected from different animal farms

during arboviral surveillance located at Xinjiang, China which also have little pig population as in Bangladesh [31]. Although the role of birds as a reservoir hosts for JEV is admitted but the role of birds as potential hosts has been poorly investigated in the past.

Unlike in pigs, no onward transmission occurs from humans because JEV induced viremia is insufficient to be infectious to the mosquito vector, making humans as dead-end host for JEV, therefore mosquitoes cannot get infection from infected person [32]. Vertebrate population density, life span and JEV viremia were considered when implicating primary hosts.

3. Discovery of birds as the natural reservoirs and amplifying host for JEV

Role of birds as reservoir hosts for JEV is admitted till 1958 [24, 33], however, the role of birds as potential amplifying hosts has been little investigated so far. Several surveys conducted in different continents suggest the involvement of domestic birds in arboviruses dispersion, especially ducks, as involved in WNV epidemiological cycle, either as amplifying hosts or as a reservoir [34-36], which is most closely related to JEV among flaviviruses and share ecological resemblance as they maintain an enzootic transmission cycle with several bird families as natural reservoirs and mosquitoes of *Cx.* species as main vector [19, 37]. With regard to JEV, a number of studies have been conducted in birds to determine the seroprevalence of JEV. For example; Saito et al., suggested that wild ducks can play an important role as a JEV reservoir hosts [38]. Saito and his colleagues captured 92 wild ducks--50 Anas platyrhynchos (undetermined), 16 Anas acuta (winter visitors), 6 Anas penelope (winter visitors), and 20 Anas poecilorhyncha (migratory breeders) in autumn of 2005 and 2006, in the central part of Hokkaido, a low JEV activity area. They performed seroepidemiologic analysis of JEV and tested 5.4% and 85.9% positive for JEV-specific antibodies with 90% (FRNT 90) and 50% focus reduction neutralization tests (FRNT 50), respectively [38]. In addition, Yang et al., reported that out of 1,316 serum samples tested, 84.7% to 88.5% sero-prevalence for JEV in wild birds

including ducks (Anas penelope, Anas formosa, Anas crecca, Anas acuta, Anas poecilorhyncha, Anas platyrhynchos), petrels (Oceanodroma castro), mandarin ducks (Aix galericulata), and Eurasian coots (Fulica atra) during 2009 from Korea [39]. In Bali (Indonesia), there were 20.6% of ducks and 36.7% of chickens were tested positive [40], and a study from Malaysia found 28.9% of the tested domestic birds positive for JEV antibodies [41]. A recent study from Cambodia reports 29% (180/620) of the domestic birds positive for flavivirus antibodies with an age-depended increase of the seroprevalence (OR = 1.04) and a higher prevalence in ducks compared to chicken (OR = 3.01) [42]. Within the flavivirus positive birds, they found 43% (28/65) with nAb against JEV [42].

Along with this, a number of recent experimental studies shows that domestic birds can be infected with JEV [24, 43-45] and might even act as JEV reservoirs [46, 47]. A recent study from Korea reports that the distribution and the density of migratory birds are correlated with JE cases in cities and they might be highly potential hosts contributing to transmit JEV in metropolitan areas [20]. Because of the bird's close association to humans and varying levels of seroprevalence observed in birds, their role in epidemiological cycle as secondary reservoirs may be of importance.

4. Species of birds susceptible to JEV infection

The inter-continental spread of JEV and other arboviruses to non-endemic areas is a continual impeding threat [48]. The circulation of JEV in the Southeast Asia is well-documented, and the important role of pigs as amplification hosts for the virus is well known from long time. However, presently pigs play a less important role as amplifying hosts as compare to past because of the JEV vaccination and vector control at farms. The influence of domestic and wild birds that lives in high abundance and close proximity to human and animals is not well studied. Similar to the unanticipated spread of WNV in America, the geographic range of JEV has expanded within the past decade.

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Species within the avian family *Ardeidae* e.g., herons, bitterns, and ergets were initially targeted for JEV research because they are seasonally abundant and available, and are relatively easy to sample. Subsequent high seroprevalence and JEV isolations from ardeids in Japan and India gives an idea that they may be have an important role in JEV dispersion [33, 49]. Characterization of avian host species response to JEV infection and seroprevalence is an important fact to elucidate the birds' role as reservoir host. Differences in JEV viremia profiles among variety of avian species were observed in a number of studies which we are discussing below.

Nemeth et al., observed variation in interspecies responses among North American birds during 2011-2012. They used 16 species of birds from eight taxonomic orders as shown in Table 1 [18] to JEV infection with genotypes I and III. Nemeth team noticed that the majority of individuals of all species inoculated with JEV genotype I or III had highest average peak viremia titers except for fish crows, ring-necked pheasants, American crows, American white pelicans, and double-crested cormorants; no individuals of these species had detectable viral load. Whereas, majority of the birds, both viremic (72 of 74; 97.3%) and non-viremic (31 of 37; 83.8%), were seroconverted by 14 days post-inoculation [18].

In 2014, Cleton et al., investigated the magnitude of virmia in 2- days old chicks and ducklings after JEV infection [45]. In their study, 2 days old chicks and ducklings were represented with peak viremia at 3 days post infection which was 4.7 (log10 plaque-forming units/mL) and 6.3 (log10 plaque-forming units/mL), respectively (Table 1) [45]. In addition, infection was associated with reduced weight gain in both species, and ducklings infected at 10 days of age or less showed overt clinical signs of disease. Furthermore, the mean peak viremia in birds of both species decreased as the age at infection increased from 2 to 42 days,

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indicating the importance of age on magnitude of viremia in birds from both species, and suggesting that young poultry birds may be amplifying hosts of importance in disease-endemic regions.

Following Cleton et al., study, we have examined the pathogenicity of JEV strains (SD12, SH1, SH2, SH7, SH15, SH19, and N28) in the Shaoxing ducklings at day 2 post hatching [43]. After subcutaneously inoculation with 10,000 plaque-forming units of JEV per bird, all ducklings were monitored for 7 days and weighed daily from 0 dpi to the end (7dpi) of experiment to calculate the average daily weight gain (ADWG). A blood samples were taken from jugular vein at 2 dpi for the detection of viremia by 50% tissue culture infectious dose (TCID50) assay. Some JEV-inoculated birds showed mild and non-characteristic clinical signs starting from 2 dpi. The ADWG of all JEV strain-inoculated ducklings was significantly lower as compare to that of mock-inoculated ducklings during the 7-day experiment, with reductions of 2.1–4.5 g suggesting stunted growth in the JEV-inoculated ducklings [43]. No death was observed in ducklings challenged with SD12, SH1, SH7, or SH15. In contrast to this, significant mortality was observed in ducklings inoculated with JEV strains N28 (31.7 %, p=0.0043) and SH19 (12.7%, p=0.0379) [43]. Furthermore, the proportions of viremic ducklings and the viremia titers differed among strains, with the highest proportion (69.2%) of viremic individuals and the highest viremia titer ($10^{3.4\pm1.3}$) in ducklings inoculated with SD12 strain (Table 1). These results suggest that the response and susceptibility of ducklings to JEV infection differed among JEV strains [43]. Along with this, previous study also reported JEV-induced death in experimentally-infected wild birds of several species [50].

In another study, Karna and his team infected 5 to 6 days of age Indian runner ducks (Anas platyrhynchos domesticus) with ~10^6 PFU of JEV using 6 different strains from JEV genotype I and III (Table 1). The mean peak viremia titer developed in inoculated ducklings at 2-3 dpi against six strains of JEV GI and GIII

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(mean ± 1 SE, log10PFU/mL) were: KE-093-83 (4.1 ± 0.2), MAR864 (3.3 ± 0.2), JE-91 (4.2 ± 0.1), CH392 (3.0 ± 0.8), JKT27-087 (4.2 ± 0.3), and Sagiyama (3.3 ± 0.6) [51]. The mean peak viremia titer (mean ± 1 SE, log10PFU/mL) in the ducklings for G-I and G-III were 3.9 ± 0.2 and 3.5 ± 0.3, respectively. However, none of the ducklings presented with signs of disease or distress [51]. Notably, in another experiment we have inoculated Shaoxing ducklings at day 2 post-hatching to compare replication efficiencies between JEV GI and GIII strains [44]. All injected ducklings were developed viremia with similar viremic rates between GI and GIII, while the viremic duration of GI-inoculated ducklings was notably, but not significantly (p = 0.0525), longer than GIII-inoculated ducklings (Table 1) [44]. These data further support that JEV infection leads to development of viremia in birds.

Results from all the above mentioned studies revealed that relatively small JEV doses injected subcutaneously into birds resulted in infection, implying that relatively low viral quantities injected into birds by mosquitoes could result in infection. However it has an important caveat that all the above mentioned investigations lack natural route of infection in birds as by mosquitoes because difference in inoculation route could leads to difference in pathogenicity of the same JEV strains. The components of mosquito saliva play roles in modulating host immune responses and in facilitating the replication and transmission of flaviviruses [52-54]. Furthermore, the amount of virus present in host blood after a bite by an infectious mosquito is also an important parameter in determining the extent to which a host may contribute to transmission [55].

Recently, we have noticed 30% mortality in newly hatched Shaoxing ducklings when bitten by infected mosquitoes under lab conditions [56], which was not seen in the domestic ducklings subcutaneously inoculated with the same JEV strain used in our previous study [43]. The infected ducklings died suddenly,

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with neurological signs of opisthotonos (a condition of spasm of the back muscles causing the head and limbs to bend backward and the trunk to arch forward) between 2 and 3 dpi. The highest RNAemia were observed in the affected ducklings at 2 and 3 days infected mosquito bite (Table 1) [56]. However, the remaining ducklings exposed to JEV-

Table 1. Summary of viral titers of birds experimentally inoculated with Japanese encephalitis virus

Birds	**age**	**Level** (log10 plaque-forming units/mL)	**reference**
Fish crow		$< 10^{1.7}$	[19]
Ring-necked pheasant		$< 10^{1.7}$	[19]
Mallard		$10^{2.0–3.3}$	[19]
House sparrow		$10^{1.7–3.7}$	[19]
Red-winged blackbird		$10^{2.3–4.0}$	[19]
Rock pigeon		$10^{2.7–4.3}$	[19]
European starling		$10^{2.5–3.6}$	[19]
House finch		$10^{3.8–4.9}$	[19]
Common grackle		$10^{3.3–4.4}$	[19]
Ring-billed gull		$10^{3.5–5.4}$	[19]
Cattle egret		$10^{2.0–3.1}$	[19]
American crow		$< 10^{1.7}$	[19]
American white pelican		$<10^{0.7}$	[19]
Double-crested cormorant		$< 10^{0.7}$	[19]
Chicken		$10^{1.7}$	[19]
Great egret		$10^{3.4–4.2}$	[19]
Chicks ¶	2 days	$10^{4.7}$	[47]
Ducklings¥	2 days	$10^{6.3}$	[47]
Ducklings (SD12)	2 days	$10^{3.2±0.7±}$	[45]

Ducklings (SH1)	2 days	$10^{2.2\pm0.7\pm}$	[45]
Ducklings (SH2)	2 days	$10^{2.8\pm0.9\pm}$	[45]
Ducklings (SH7)	2 days	$10^{2.5\pm0.6\pm}$	[45]
Ducklings (SH15)	2 days	$10^{2.0\pm0.4\pm}$	[45]
Ducklings (SH19)	2 days	$10^{2.9\pm0.8\pm}$	[45]
Ducklings (N28)	2 days	$10^{2.5\pm1.1\pm}$	[45]
Ducklings (KE-093-83)	5-6 days	$10^{(4.1 \pm 0.2)}$	[53]
Ducklings (MAR864)	5-6 days	$10^{(3.3 \pm 0.2)}$	[53]
Ducklings (JE-91)	5-6 days	$10^{(4.2 \pm 0.1)}$	[53]
Ducklings (CH392)	5-6 days	$10^{(3.0 \pm 0.8)}$	[53]
Ducklings (JKT27-087)	5-6 days	$10^{(4.2 \pm 0.3)}$	[53]
Ducklings (Sagiyama)	5-6 days	$10^{(3.3 \pm 0.6)}$	[53]
Shaoxing ducklings (SH2)	2 days	0.80ȧ	[46]
Shaoxing ducklings (SH7)	2 days	2.25 ȧ	[46]
Shaoxing ducklings (SD12)	2 days	0.2 ȧ	[46]
Shaoxing ducklings (N28)	2 days	0 ȧ	[46]
Shaoxing ducklings (SH1)	2 days	0 ȧ	[46]
Shaoxing ducklings (SH15)	2 days	0.2 ȧ	[46]
Shaoxing ducklings (SH19)	2 days	0 ȧ	[46]
Ducklings (mosquito bite)	2-3 days	$3x10^4$ µ	[58]
Ducklings (mosquito bite)	2-3 days	$3x10^5$ Δ	[58]
Chicken (GIII CH1392)	1 days	4.75€	[76]
Chicken (GIII T1P1)	1 days	5.10€	[76]
Chicken (GI YL2009-4)	1 days	6.0€	[76]
Chicken (GI TC2009-1)	1 days	6.25€	[76]
Ducklings (GIII CH1392)	2 days	4.10€	[76]
Ducklings (GIII T1P1)	2 days	3.40€	[76]
Ducklings (GI YL2009-4)	2 days	4.30€	[76]

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Ducklings (GI TC2009-1)	2 days	4.30€	[76]

¶, values of highest viral titer after 3 dpi in 2 day old chicks

¥, values of highest viral titer after 3 dpi in 2 day old ducklings

±, Viremia titer was tested by $TCID_{50}$ assay ($TCID_{50}$/0.1ml) at 2 dpi

µ, Copies of JEV E gene at 2 days post infected mosquito bite

∆, Copies of JEV E gene at 3 days post infected mosquito bite

€, FFU titer at 2 days post infection

ά TCID50 viral titer measured at 4 days post infection

infected mosquitoes showed no noticeable clinical signs. This apparent difference in the pathogenicity of the same JEV strain may be attributable to the difference in the inoculation route between the two experimental challenges. These observations indicated that JEV infection *via* mosquito bite causes mortality associated with viral encephalitis in newly hatched ducklings, thus demonstrating the potential pathogenicity of JEV in domestic ducklings under natural conditions. The virmia found specifically in the young birds were high enough that can resulted in 50-100 % transmission in *Culex* mosquitoes [57, 58], which are known competent vectors for JEV [25].

Considering these estimates for the vector competence, these studies suggest that efficient transmission of JEV to mosquitoes likely occurs from young chicks, ducklings and other bird species, may play an epidemiologically significant role in JEV transmission. Bird's potential for JEV to spread to non-endemic areas and potential impact of particular farming systems, including duck farming needed further investigation. Along with this, field studies to explore the force of infection in these hosts during JEV transmission events are necessary to further validate their role in the JEV transmission dynamics.

5. Pathogenicity of JEV in domestic birds

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JEV-induced mortality were reported in experimentally-inoculated wild birds of several species [50]. Previous experiment data show that inoculation of domestic ducklings with different JEV strains resulted in overt clinical signs, stunted growth and variable viremia in all JEV-inoculated ducklings [43, 45], suggesting the potential pathogenicity of JEV in newly hatched domestic ducklings. Although no JEV-related outbreaks have yet been reported in domestic ducklings, but recent findings suggest that the responses and susceptibilities of ducklings to JEV infection is age dependent and differ among JEV strains. Among the seven JEV strains used by Xiao and his colleagues, only one showed high virulence in ducklings, and a proportion of ducklings failed to develop detectable viremia after JEV inoculation, suggesting that most JEV strains circulating in natural hosts might have low or nonexistent pathogenicity in domestic ducklings. In addition, natural JEV infection *via* JEV-infected *Cx. pipiens* mosquito bites in newly hatched domestic ducklings caused 30% mortality, that were associated with viral encephalitis [56]. These observations demonstrated the potential pathogenicity of JEV in domestic ducklings under natural conditions. Presently, it is also possible that any JEV outbreak in ducklings might be ignored or misdiagnosed because of the mild and non-characteristic clinical signs and the relatively low mortality. In future, surveillance of ducklings dying in the mosquito season in JEV-endemic areas is needed to elucidate the potent pathogenicity of JEV in poultry birds and possible prophylactic strategies we should take to avoid its outbreaks.

6. *In vitro* and *in vivo* studies shows the potential role of birds in JEV genotype shift

GIII was an endemic strain in Asia but recently, GI has displaced GIII as the most frequently isolated virus genotype. The exact mechanism that leads to this genotype shift is still not clear. In past, there have been a number of reports about the isolation of JEV GI from human, mosquitoes, and pig samples. A recent study

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reported the JEV outbreak among human caused by GI in Ningxia in the Northern China [59]. This study confirmed the JEV GI outbreak by isolating G I from laboratory (Human) and field data (mosquitoes).

Cx. mosquitoes mostly play an important role in the transmission of JEV in all over the endemic regions. Recently, we have studied *Cx.pipiens* mosquitoes role in JEV genotype shift [25]. Our experiment data demonstrated that GI and GIII viruses have similar infectivity in *Cx*. *pipiens* mosquitoes, suggesting that mosquitoes may not play a critical role in JEV genotype shift.

Birds play an important role in the maintenance and transmission of many arboviruses including JEV. Quantifying the relative contributions of bird's species involved in JEV transmission, and the role of birds in particular, would improve assessments of the potential for JEV to spread to new geographic regions [30, 48, 60], role in genotype shifting, and the potential impact of particular farming systems, including duck farming in rice paddies.

As we have discussed above that avian species can develop viremia after either natural exposure or challenged in lab [61-63]. These reservoir hosts may have some important role in genotype shift that can be explored. In a previous study, we observed that JEV GI strains replicated more efficiently than GIII strains particularly in birds derived cells and in the young ducklings [43, 44]. This shows that JEV GI has an advantage in replication efficiency and host adaptation in birds which can lead to JEV genotype shift. However, the mechanism behind this adaptation in birds required further investigation.

In a recent study from Taiwan, Fan Y-C and his coworkers compared JEV GIII and GI infectivity in one day old chickens and two day old ducklings [64]. Fan Y-C et al.,inoculated 10^4 FFU of GIII and GI viruses in one day old chicks and two day old ducklings. They observed 100% (8/8) and 75% (6/8) viremia in GI infected chicks and ducklings (Table 1). Whereas, 37.5% (3/8) and 12.5% (1/8) of GIII inoculated chicks

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and ducklings had developed viremia [64]. Their data demonstrated that JEV GI infected birds showed a significantly ($p<0.05$) higher viral titer (0.60-1.73-log) as well as earlier and long lasting viremia than birds inoculated with GIII strains as shown in Table 1.

They have performed a series of experiments by using chimeric viruses (pCMV GIII/GI UTR, pCMV GIII/GI C-E, pCMV GIII/GI NS1-5, pCMV GIII/GI NS1-3, pCMV GIII/GI NS4-5) and demonstrated that the higher GI virus infectivity determinants are present in the NS1-3 genes of the JEV genome. They further verify the specific substitutions of GI NS1-3 protein by introduction of a single virus specific and highly consensus substitution in rGIII/GI NS1-3 chimeric viruses. Their experimental data concluded that the GI residues NS2B-V99L and NS3-A78S, NS3-E177D were involved in the replication enhancement of GI virus *in vitro* (DF-1) and *in vivo* (1 day old chicks).

Recently, we had conducted a deep investigation to identify the viral determinants of differing multiplication capability between GI and GIII viruses in birds. We examined the difference in Interferon –I (IFN_I) stimulation between GI and GIII by using duck embryo fibroblasts (DEF) and domestic ducklings as an *in vitro* and *in vivo* avian models, respectively [65]. The DEF, mouse endothelial cell line (bEnd.3), and swine testicular cells (ST) were infected with GI (SH7 and SD12) and GIII (SH15 and SH19) strains to analyze the induction of IFN- α and β expression. INF- α and β production was significantly lower in DEF cells infected with GI strains as compare to GIII viruses. Whereas, no significant difference was seen in the IFN- α and β expression in the ST and bEnd.3 cells infected with GI and GIII strains. This species-specific IFN expression by GI and GIII viruses was also confirmed by infection of duck kidney cells (DEK), porcine iliac endothelium cell line (PIEC), and mouse embryonic fibroblast cell line (MEF). Similarly, GI strains

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decreased the IFN expression in DEK cells, whereas there was no statistical difference seen in PIEC and MEF cells after GI and GIII infections. GI strains capability to stimulate low levels of IFN- α and β expression was further confirmed in the domestic ducklings. GI viruses produced viral titers 0.5-1 log higher than GIII in DEF cells due to IFN-I mediated antiviral response [66, 67].

We had used a series of chimeric recombinant viruses with the exchange of structural and non-structural proteins between the GI and GIII strains, and identified NS5 gene as the viral determinant of the differences in IFN- α and β expression and replication efficiency between the JEV strains in ducklings. GI and GIII viruses genetic analysis reveals that NS5 gene contained a total of 11 amino acid variations. We performed a series of chimeric substitution mutations and identified that NS5-V372A and NS5-H386Y variations co-contribute to the differences in IFN- α and β expression induction and replication efficiency between the JEV GI and GIII strains in DEF cells and ducklings. Then we investigated the role of NS5-372 and NS5-386 substitutions in GI and GIII strains conformation.

The substitution analysis revealed that NS5-372 makes two hydrogen bonds and NS5-386 form one hydrogen bond in GI strains with their neighboring residues, respectively. On the other hand, GIII NS5-372 substitution makes three hydrogen bonds and NS5-386 leads to the formation of two hydrogen bonds. This bonding difference in GI and GIII strains potentially result in the variation of flexibility of the NLS (nucleus locating signals) region, ultimately lead to changes in interactions with the host cell proteins and IFN-I production and viral replication. The differences in replication efficiencies, IFN-α and β production among GI and GIII strains were detected only in duck derived DEF and ducklings, but not in pig derived ST cells and mouse derived bEND.3 cells. There are two possible reasons of this difference; first the changes in the antiviral immune response between birds and mammals, and second the differences in adaptability of GI and

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GIII viruses to the IFN-I mediated antiviral response of birds and mammals. The NS5-V372A and NS5-H386Y variations allowed GI viruses to adapt to the IFN-I mediated antiviral immune response of birds, but not mammals, thereby leading to the replication advantages of GI strains over GIII in birds. These results explain the host specific differences in the IFN-I induction among GI and GIII strains leads to the replication and host advantages of GI over GIII viruses in birds, that might be a cause of JEV genotype shift. Overall, our current knowledge about the role of birds in JEV genotype shift suggest that it is important to continually monitor JEV GI virus evolution and role of birds in local transmission of JEV GI viruses.

7. Role of JEV vaccines

Although the introduction of JEV inactivated and live attenuated vaccines had dramatically reduced the JE cases. However, JEV still remains a leading cause of viral encephalitis globally. Recent detection of more divergent JEV genotype V (8.4% to 10.0% amino acid divergence compared to genotype I-IV) from China [14], Korea [13], and Malaysia [12] is threatening because it may be covered poorly by presently used JEV GIII strain based vaccines.

Mostly, JEV vaccine applied at all pig farms as a regular vaccine campaign. Therefore, quantifying the relative contributions of pigs and domesticated birds to JEV transmission is required for understanding the recent JEV ecology in regions where the pigs mostly vaccinated or pig population density is relatively low compared to the birds' population density. As we have discussed above that ducks, chickens, pigeons, and other birds produced viremia following JEV infection demonstrates their role as a JEV amplifying/reservoir hosts [18, 24, 43-45, 51, 56, 57, 68]. JEV infection produced viremia in these birds which is sufficient to infect mosquitoes, but their contribution to the JEV transmission remains to be quantified.

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We are proposing a hypothesis that should be evaluated as shown in Fig 2; (i) presently pigs contribute less due to wide application of JEV vaccines at pig farms than birds to JEV transmission and genotype shift, (ii) JEV GI shows higher replication efficiency than GIII in duck derived cells and in ducklings/chicks. Due to lack of JEV vaccination, birds might play an important role in JEV transmission and genotype shift. There are, however, currently insufficient data to fully assess this hypothesis and further study is required.

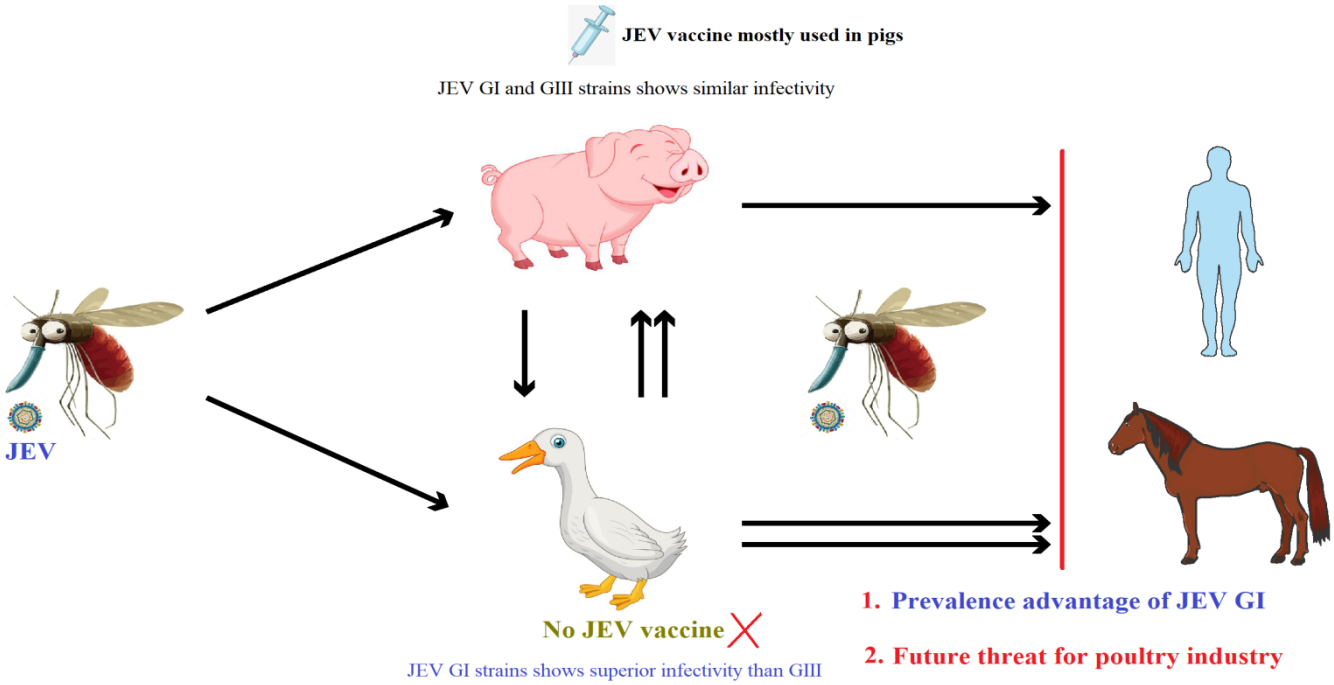

Figure 1. Overview of JEV transmission cycle and the expected role of the JEV vaccine application.

→ Shows that there is less chances of JEV transmission from pigs to birds/human/horses.

⇉ Showing that there are higher chances of JEV transmission from birds to human/horses/pigs by mosquito vectors.

8. Concluding remarks

JEV has become a significant global pathogen which is causing major public health problems in Asia. Overall, in past the role of pigs in the JEV epidemiology investigated deeply as these are well-known

amplification hosts for this virus. However, the contribution of birds to the JEV transmission remains ignored. Previous studies reported that avian species can develop viremia after either natural exposure or challenged in the lab [61-63] and can develop clinical signs, which are ubiquitous often share urban and suburban habitat with the human and mosquitoes. These amplifying/reservoir host may have some important role in the expansion of JEV affected areas which needs further investigation.

Dominant genotype of JEV has changed from III to I around 1990, and the mechanism behind this genotype shift is still unknown. Recent studies demonstrated that GI had superior replication activity in birds' derived cells as well as in young ducklings and chicks. The proposed molecular mechanism is the variation of NS2B-V99L, NS3-A78S, NS3-E177D and NS5-V372A and NS5-H386Y genes among GI and GIII viruses. These substitutions allowed GI viruses to adapt the IFN-I mediated antiviral immune response of birds, but not mammals, thereby leading to the replication advantages of GI strains over GIII in the birds. Further investigation is required to explore that how these variations enable GI viruses to inhibit IFN-I production, while GIII strains failed to do this in the birds. This also emphasizes the need for further and intensified monitoring of JEV GI evolution in birds. In addition, surveillance of JEV in backyard domestic poultry and migratory birds that serve as potential amplification hosts is required which will help focusing preventive measures, such as vaccination and vector control, in the future.

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