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FACTORES ASOCIADOS A LA VIRULENCIA DE CEPAS EPIDÉMICAS DEL VIRUS ESTOMATITIS VESICULAR NEW JERSEY

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PRESENTA:
LAURO VÉLAZQUEZ SALINAS

TUTOR
ANTONIO VERDUGO RODRÍGUEZ
FACULTAD DE MEDICINA VETERINARIA Y ZOOTECNIA

COMITÉ TUTOR
LUIS L. RODRÍGUEZ
PLUM ISLAND ANIMAL DISEASE CENTER

RAMÓN GONZÁLEZ GARCÍA CONDE
UNIVERSIDAD AUTÓNOMA DEL ESTADO DE MORELOS

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Dedicatorias

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“Por mi raza hablará el espíritu”

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Índice

RESUMEN

El objetivo de esta tesis fue demostrar que cepas epidémicas del virus de estomatitis vesicular New Jersey (VSNJV) podrían representar un fenotipo más virulento para el cerdo que sus parientes endémicos más cercanos. Para este fin, se utilizaron como modelo experimental las cepas del VSNJV: NJ0612NME6 (linaje epidémico 1.1) y su pariente endémico más cercano la cepa NJ0806VCB (linaje 1.2), con las cuales se realizaron estudios de patogenicidad en cerdos, análisis de comparación genómica, así como experimentos de expresión génica y cinéticas de crecimiento en diversas líneas celulares. El estudio comparativo de patogénesis en cerdos, mostro que la cepa NJ0612NME6 representaba un fenotipo más virulento que la cepa NJ0806VCB, sugiriendo que una de las principales diferencias biológicas entre ambos virus, podría ser la habilidad del virus NJ0612NME6 para interferir con la respuesta inmune innata en el cerdo. Por otro lado, los resultados de la comparación genómica revelaron un patrón complejo de substituciones nucleotídicas, indicando que diferentes proteínas en el VSNJV podrían ser responsables de las diferencias en la virulencia entre ambas cepas. Posteriormente, con la finalidad de obtener más información sobre potenciales factores biológicos que podrían estar influenciando la virulencia del VSNJV en los cerdos, se realizó mutagénesis dirigida en la clona de DNA NJ0612NME6 (creada como parte de esta tesis), para desarrollar la mutante M51R, la cual representa un fenotipo viral altamente deficiente en su habilidad para controlar la respuesta inmune innata. De manera interesante, los resultados de la caracterización biológica llevados a cabo *in-vivo* e *in-vitro* utilizando la mutante M51R, indicaron que los ambientes celulares inmunes y no inmunes podrían representar retos diferentes para el VSNJV durante la infección en cerdos. En este contexto, se reporta la existencia de una correlación positiva entre la habilidad del VSNJV para replicar en cultivos primarios de macrófagos y el nivel de virulencia mostrado en el cerdo. En conclusión, los resultados de esta tesis muestran por primera vez que las cepas epidémicas del VSNJV podrían representar un fenotipo mas virulento para los cerdos, en comparación son sus parientes endémicos mas cercanos. Asimismo, se identificaron a los cultivos primarios de macrófagos porcinos, como un blanco para la realización de estudios futuros en la determinación de interacciones virus-hospedador asociadas con la virulencia de VSNJV

en cerdos. Subsecuentes estudios son necesarios para confirmar que las cepas epidémicas del VSNJV representan un fenotipo más virulento en diversos animales domésticos involucrados en el ciclo infeccioso de este virus, que sus parientes endémicos más cercanos, así como la significancia de este hallazgo durante epizootias. Adicionalmente, los resultados revelaron aspectos nuevos sobre la patogenia del VSNJV en cerdos, por ejemplo, la identificación de una fase de RNAemia durante la etapa aguda de la infección, la cual podría ser usada en futuros estudios como marcador indicativo de la virulencia de las diferentes cepas de VSNJV. Igualmente, más estudios son necesarios para entender los factores intrínsecos y extrínsecos asociados con la virulencia de VSNJV en animales domésticos, centrándose en determinar interacciones virus-hospedador en fenotipos celulares inmunes y no inmunes.

Abstract

The goal of this thesis, was to demonstrate that the epidemic strains of vesicular stomatitis New Jersey Virus (VSNJV), might represent a more virulent phenotype for pigs than its endemic relatives. For this propose, VSNJV strains NJ0612NME6 (epidemic lineage 1.1), and its closest endemic relative, the strain NJ0806VCB (lineage 1.2) were used as experimental models to conduct pathogenesis studies in pigs, analysis of genomic comparison, as well as experiments of gene expression and growth curves in different cell lines. The comparative pathogenesis study in pigs showed that NJ0612NME6 virus represents a more virulent phenotype for pigs than NJ0806VCB virus, suggesting that one of the main biological differences between the viruses might be the ability of NJ0612NME6 to interfere with the innate immune response in pigs. On the other hand, The results of the genomic comparison reveled a complex pattern of nucleotide substitutions, indicating that different proteins in the VSNJV might be responsible for the differences in the virulence between strains. In order to gain more insight on the potential biological factors leading the virulence of VSNJV in pigs, we used reverse genetics in a DNA clone of NJ0612NME6 (created as a part of this thesis) to developed the mutant M51R, a viral phenotype highly defective in its ability to overcome the innate immune response. Interestingly, results from our in-vitro and in-vivo characterization indicate that non-immune and immune cellular environments might represent different challenges for VSNJV during the infection in pigs. In this context, we are reporting a positive correlation between the ability of VSNJV to replicate in primary macrophages cell cultures and the virulence in pigs. In conclusion, the results of this thesis show for the first time that epidemic strains of VSNJV might represent a more virulent phenotype for pigs than its endemic relatives. Also, the results point to porcine macrophages as a target for future studies on virus-host interactions determining the virulence of VSNJV in pigs. Consequent studies are necessary to determine the relevance of these findings in other domestic species involved in the infectious cycle of VSNJV, and to understand its significance during epizootics. Additionally, our results reveal new insights about the pathogenesis of VSNJV in pigs, for example by identifying for the first time a phase

of RNAemia during early the early stages of the infection, a phase that might be used as a biological marker of virulence for future comparative studies of virulence in pigs. Likewise, more studies are needed to gain a better understanding of the intrinsic and extrinsic factors leading to the virulence of VSNJV in livestock. In this sense, strategies to conduct new studies should be focused on determining virus-host interactions of VSNJV at both non-immune and immune cellular phenotypes.

CAPITULO 1

INTRODUCCIÓN

1.1 Generalidades

La estomatitis vesicular (VS) es una enfermedad viral, la cual es causada por el virus de la estomatitis vesicular (VSV), un virus perteneciente a la familia *Rhabdoviridae* y al género *vesiculovirus*, del cual se han descrito dos serotipos principales: Indiana *vesiculovirus* (VEVID) y New Jersey *vesiculovirus* (VEVNJ) [1]. En el caso del VEVID, este se encuentra clasificado en cuatro diferentes complejos serológicos: VEVID 1, VEVID 2 (Cocal vesiculovirus), VEVID 3 (Alagoas vesiculovirus) y VEVID 4 (Morreton vesiculovirus) [2].

La circulación del VSV se encuentra confinada exclusivamente al continente americano, donde el virus circula de manera endémica en regiones tropicales, y subtropicales de este continente, presentando una circulación epizoótica ocasional (intervalos 6-10 años) en zonas templadas de los Estados Unidos, iniciando la presentación de brotes clínicos durante los meses de verano [1, 3]. A nivel epidemiológico, el VEVNJ es el serotipo más importante , al ser responsable de 80% de casos clínicos reportados anualmente en América [1, 4, 5] .

VS es una enfermedad auto limitante que afecta animales domésticos incluidos: bovinos, equinos, porcinos y en menor grado caprinos y ovinos; y se caracteriza por la producción de lesiones vesiculares en encías, lengua, paladar, labios, trompa, patas, glándulas mamarias y prepucio, las cuales pueden complicarse por infecciones secundarias con bacterias, y conducir a la presentación de cuadros clínicos de mastitis [3]. Asimismo, las lesiones vesiculares producidas por esta enfermedad son clínicamente indistinguibles a las producidas por el virus de la fiebre aftosa, uno de los virus con mayor impacto económico a nivel mundial en los animales domésticos [1]. Debido a esta similitud, la EV es considerada una enfermedad de reporte obligatorio y hatos afectados deben ser puestos obligatoriamente en cuarentena, hasta que la ausencia del virus de la fiebre aftosa en las muestras clínicas sea confirmada por pruebas de laboratorio [1].

Sin embargo, más allá de su similitud clínica con la fiebre aftosa, VS tiene la capacidad de producir grandes pérdidas económicas provocadas no solo por la baja severa de peso en los animales afectados, la cual tiene un impacto profundo en la producción láctea bovina, sino también, por la imposición de cuarentenas, que en el caso de los equinos puede producir cuantiosas pérdidas económicas debido a la cancelación de presentaciones en diferentes tipos de espectáculos ecuestres [1, 6, 7]. Las pérdidas económicas en los Unidos durante la presentación de eventos epizoóticos, han sido estimadas en aproximadamente 16,000 dólares por rancho afectado [3, 8, 9].

En México, la VS es considerada como endémica en los estados de Veracruz, Tabasco y Chiapas, los cuales concentran la mayor diversidad genética del VSV en este país [4, 10, 11]. En estos estados, la presentación de casos clínicos se reporta de manera anual, con un mayor índice de casos reportados durante los meses de septiembre, octubre y noviembre [11]. Así mismo, el análisis genómico de los virus aislados en estos estados mostró que los casos clínicos son causados por múltiples linajes genéticos establecidos endémicamente en estas zonas [4]. En este contexto, estudios filogenéticos utilizando la región hipervariable del gen de la proteína P como marcador evolutivo, han estimado la existencia de por lo menos 9 linajes virales y una variabilidad genética de aproximadamente 4% en México [11].

Por otro lado, esporádicamente se suscita la emergencia de linajes virales con la capacidad de diseminarse fuera de las zonas endémicas, produciendo casos clínicos en las zonas centro y norte de México, y eventualmente provocando brotes epidémicos en el sur de los Estados Unidos, con una duración de hasta por 5 años [1, 12, 13, 4]. Actualmente las causas asociadas con la emergencia de estos linajes, son completamente desconocidas.

Con base en la clasificación filogenética propuesta por Pauszek y colaboradores en el 2012 [24,] típicamente los linajes genéticos del VEVNJ responsables de la producción de casos clínicos en México y los Estados Unidos están asociados al clado I (virus circulantes en Norteamérica). Sin embargo, recientemente se reportó la presencia de linajes genéticos pertenecientes al clado II (virus circulantes en Centroamérica) dominando las infecciones clínicas en el ganado en las zonas sur y centro de México entre el 2008 y el 2012 [4] (Figura

1), lo que sugiere el potencial de estos linajes para causar epizootias en el norte de México y sur de los Estados Unidos.

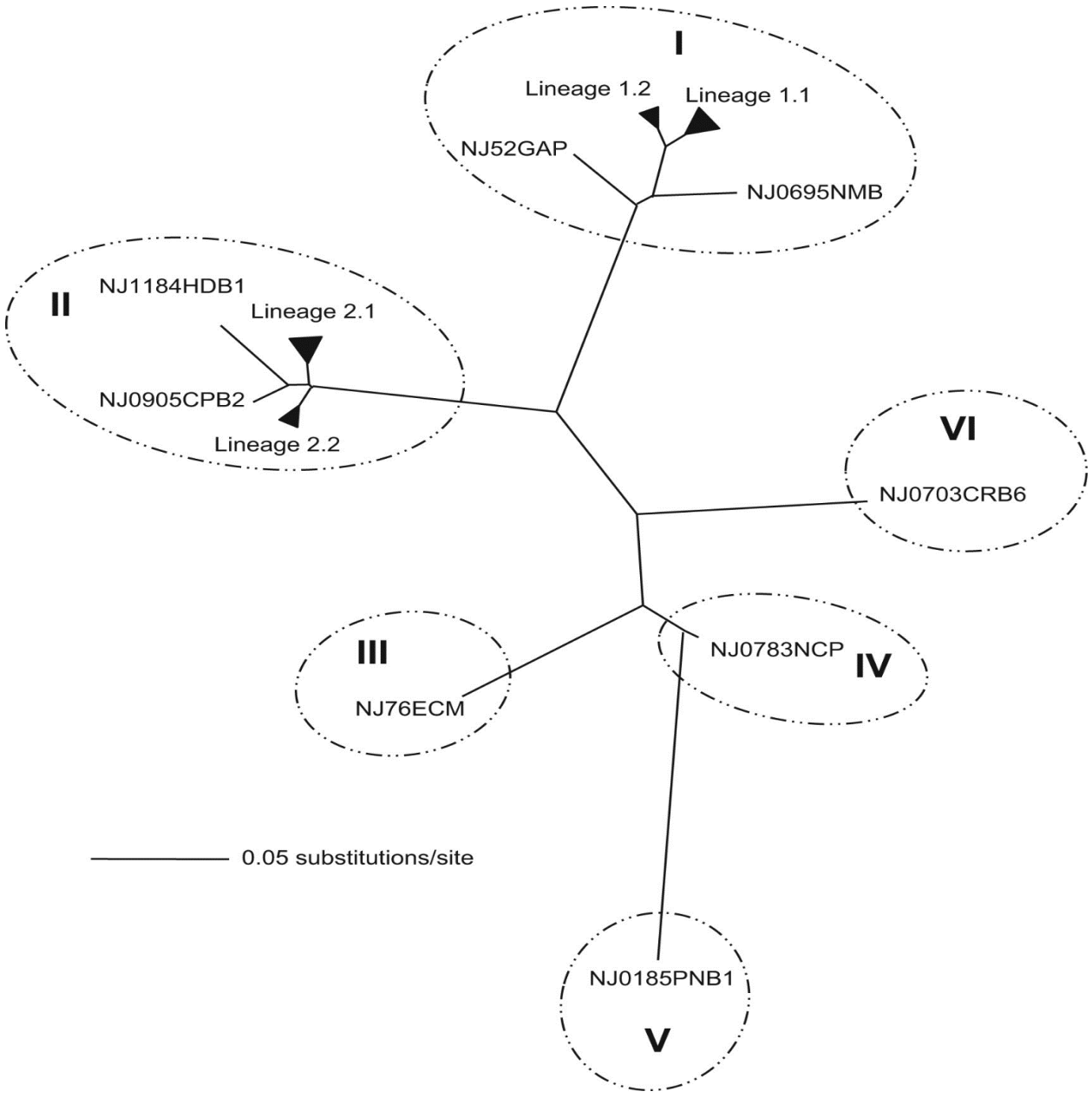

Figura 1. Árbol filogenético reconstruido por el método de *Neighbor-joining* usando la región hipervariable del gen P, el cual muestra la clasificación filogenética del VSNJV propuesta por Pauszek y colaboradores[24]. En el clado I (Norteamérica) se encuentran los virus típicamente circulantes en México y los Estados Unidos, como es el caso de los linajes 1.1 y 1.2. Recientemente se identificó en México la circulación de un grupo de linajes (2.1, 2.2) pertenecientes al clado II (Parte norte de Centroamérica).

Fuente: Velazquez-Salinas L, et al Virology. 2014; 449:17-24 [4]

La capacidad del VSNJV para emerger y producir eventos epizoóticos esporádicos en el sur de los Estados ha sido un fenómeno ampliamente documentado durante las últimas tres décadas [1, 12, 13, 4]. A pesar de que las causas atribuibles a la emergencia del VSNJV en los Estados Unidos son desconocidas, la estrecha relación filogenética entre las cepas responsables de los brotes epidémicos en este país y las circulantes en las zonas endémicas de México, han permitido proponer la hipótesis, de que la reemergencia de la estomatitis vesicular (VS) en los Estados Unidos es producto de la introducción de cepas virales provenientes de zonas geográficas ajenas a los Estados Unidos, más que de la evolución de cepas virales a partir de ancestros endémicos potencialmente establecidos en este país [4].

Esta hipótesis encontró sustento en la tesis de maestría titulada "Epidemiologia molecular del virus de estomatitis vesicular serotipo New Jersey en México periodo 2005-2009", donde se evidencia por primera vez, la habilidad del linaje epidémico denominado 1.1, para diseminarse de manera progresiva México en dirección centro-norte entre los años 2006 y 2009, para finalmente ser aislado en el sur de los Estados unidos durante el año 2012 donde permaneció por 5 años (Figura 2). Los análisis filogenéticos realizados utilizando las secuencias de cerca de 200 cepas virales colectadas en México y los Estados Unidos entre los años 2005 y 2012, mostraron la estrecha relación genética del linaje epidémico 1.1 con el linaje endémico 1.2, el cual comprende un grupo de cepas virales, las cuales circularon de manera endémica en el estado de Veracruz entre los años 2006 y 2008 [4] (Figura 3).

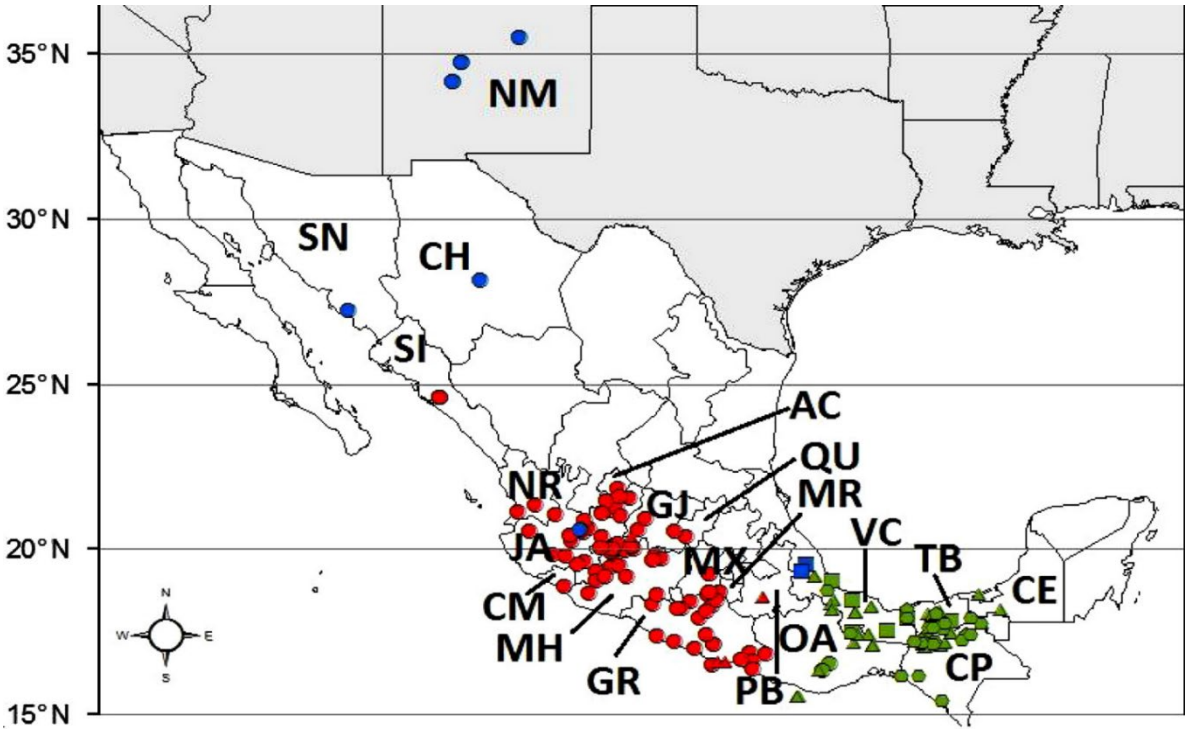

Figura 2. Análisis de clúster geográfico elaborado con 181 aislamientos virales del VSNJV colectados en México y los Estados Unidos entre 2005 y 2012, mostrando la ruta de circulación del linaje epidémico 1.1. En colores rojo y verde se muestran las zonas geográficas con mayor probabilidad de circulación viral en México. En Azul se representan zonas con menor probabilidad. En círculos esta representado el linaje epidémico 1.1, mientras que, en cuadrados se representa al linaje 1.2, su ancestro endémico más cercano. Las otras formas geométricas representan diversos linajes virales encontrados en México durante el periodo de muestreo de este estudio.

Fuente: Velazquez-Salinas L, et al Virology. 2014; 449:17-24 [4]

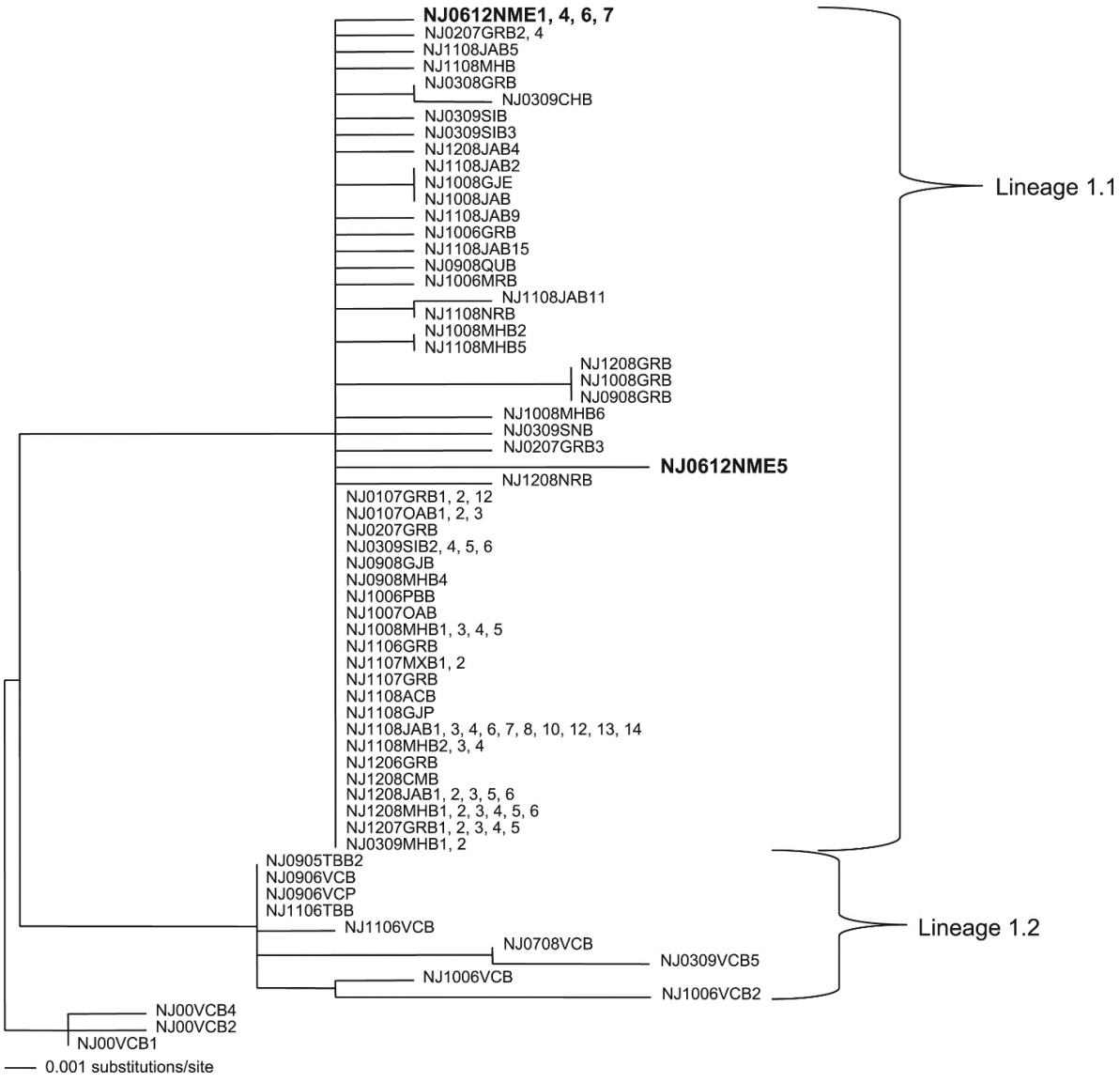

Figura 3. Análisis filogenético elaborado por el método de máxima verosimilitud, usando la región hipervariable del gen P, el cual muestra la relación genética entre el linaje epidémico 1.1 y su pariente endémico más cercano, el linaje 1.2.

Fuente: Velazquez-Salinas L, et al Virology. 2014; 449:17-24 [4]

1.2 ORGANIZACIÓN GENÓMICA Y PROTÓEMICA:

El genoma del VSV es del tipo RNA de cadena sencilla, con una longitud aproximada de 11 kb y de polaridad negativa (orientado en dirección 3'-N-P-M-G-L-5'). El genoma incluye cinco genes principales los cuales codifican para la producción de proteínas estructurales: gen N (nucleoproteína), gen P (fosfoproteína), gen M (proteína de matriz), gen G (glicoproteína) y el gen L (polimerasa); y adicionalmente un par de proteínas no estructurales denominadas C y C', las cuales son codificadas a partir de un segundo marco de lectura presente en el gen P [25, 26, 27]. La alta conservación de las proteínas C y C' entre los vesiculovirus, sugiere que podrían tener un papel importante en la patogenia de estos virus [27]. En los extremos del genoma se encuentran las secuencias denominadas 3' líder y 5' seguidora. Estas secuencias son complementarias en sus extremos y contienen regiones promotoras para para iniciar la replicación del genoma y antigenoma, respectivamente. Asimismo, cada uno de los genes se encuentran flanqueados por secuencias altamente conservadas que promueven la de iniciación, terminación y poliadenilación durante la transcripción [27].

En general, las partículas virales del VSV tienen la forma de bala característica de la familia *Rabdoviridae*, y presentan una dimensión aproximada de 170 nm de largo por 80 nm de ancho[28] (Figura 4). Algunas características de las proteínas del VSV se mencionan a continuación:

Nucleoproteína

La nucleoproteína (proteína N) (422 aa), es el componente más abundante de la nucleocápside viral, el cual interactúa directamente con el RNA viral para protegerlo de la acción de las enzimas RNAasas, y junto con la fosfoproteína (proteína P) y la polimerasa (proteína L) forman el complejo de replicación denominado ribonucleoproteína. La proteína N se encuentras siempre asociada con genoma viral tanto en sentido negativo como en sentido positivo (como resultado de la replicación), pero nunca con los ARN mensajeros producidos durante la transcripción. La producción de esta proteína durante la infección viral es crítica para modular los procesos de trascripción y replicación [27].

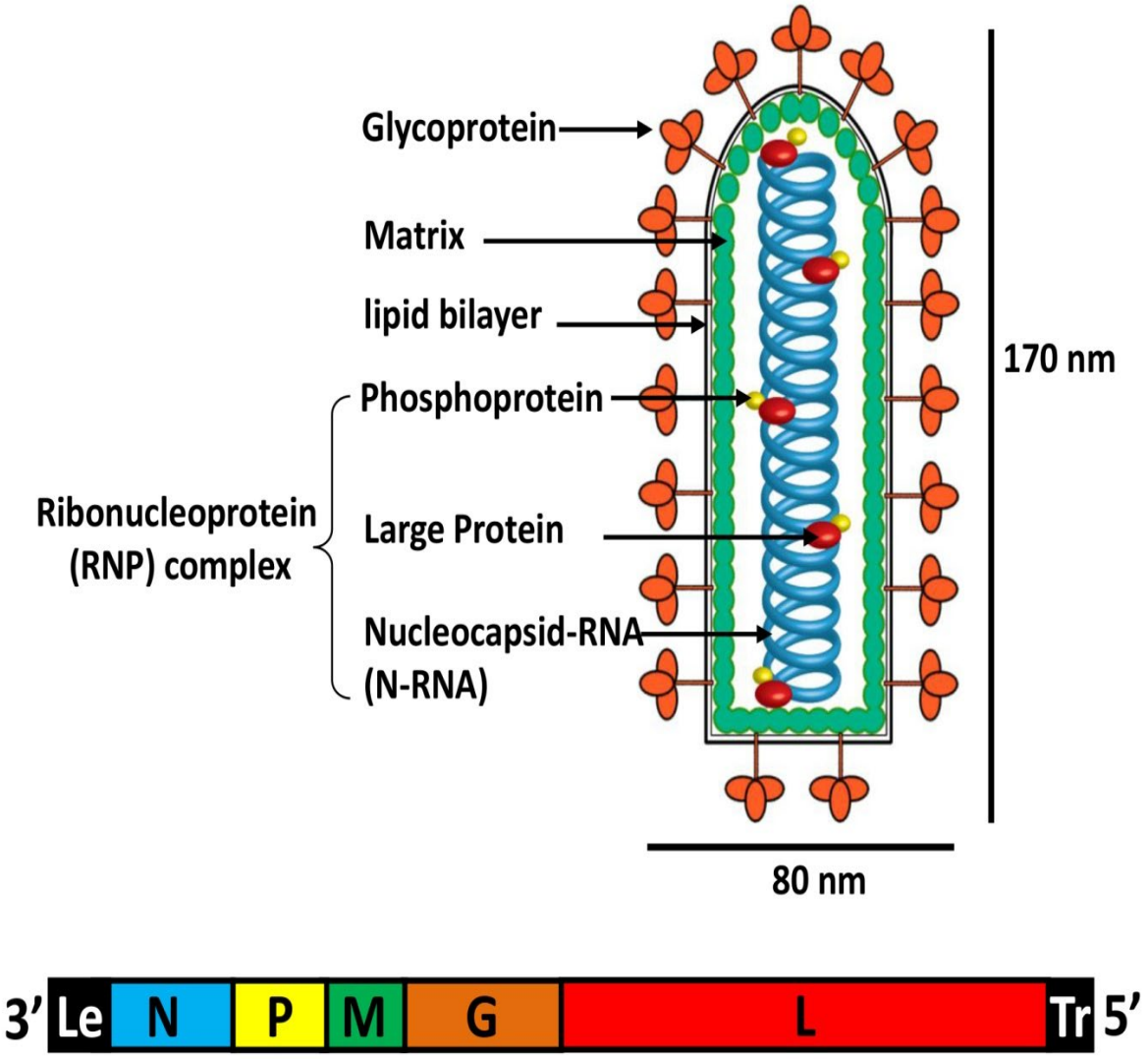

Figura 4. Organización estructural y genómica del virus de la estomatitis vesicular (VSV) [34]. El VSV esta formado por 5 proteínas estructurales: Nucleoproteína (N), fosfoproteína (P), proteína de matriz (M), glicoproteína (G) y polimerasa (L). Las proteínas N, P y L forman el complejo de replicación denominado ribonucleoproteína. El genoma se encuentra orientado en sentido negativo (3'- 5').

Fuente: Li, J. and Zhang, Y. InTech. 2012, doi: 10.5772/54598 [34]

Fosfoproteína

La fosfoproteína (proteína P) (274-265 aa) es una proteína esencial para los procesos de replicación y la transcripción viral. Esta proteína es un cofactor no catalítico de la proteína L, y se encarga de mediar la interacción física entre las proteínas L y N para formar el complejo de replicación. Asimismo, la proteína P se encarga de prevenir la unión de las nuevas proteínas N con el RNA celulares, mediante la formación de complejos con la proteína N, lo que asegura la encapsidación correcta de los genomas virales [27].

Proteína de matriz

La proteína de matriz (proteína M) (229 aa), es un componente interior y del virion, la cual tiene una función clave en el control de la respuesta inmune del hospedador. Asimismo, esta proteína, está vinculada a la inducción de apoptosis, la producción de efecto citopático durante la infección celular, y a los procesos de ensamblaje y salida del virus, al interactuar con las proteínas N y G (glicoproteína) [27].

Glicoproteína

La proteína G (511-517 aa) se ensambla en trímeros formando prolongaciones al exterior. Esta proteína permite que el VSV sea capaz de infectar un gran número de tipos celulares incluidas en organismos vertebrados e invertebrados. De igual manera, esta proteína está involucrada en los pasos iniciales de la infección que incluyen: la unión al receptor celular del hospedador y la fusión con la membrana endosomal [27, 29].

Polimerasa

La proteína L (polimerasa) (2109 aa) cuenta con múltiples dominios, los cuales permiten a esta proteína llevar acabo los procesos de transcripción y replicación, así como poliadenilación, añadido y modificación de caperuzas en los ARN mensajeros y actividades enzimáticas como quinasa, produciendo la fosforilación de residuos en la proteína P [27, 30].

1.3 CICLO DE REPLICACIÓN

En cuanto al receptor celular usado por el VSV durante la infección celular, recientes investigaciones indican que, la familia de las lipoproteínas de baja densidad, podrían ser los principales receptores en la superficie celular durante la infección [31]. Posteriormente, una vez que la proteína G hace contacto con el receptor celular, el virus es introducido a la célula por endocitosis mediada por clatrina [32] (Figura 5).

Una vez en el endosoma celular, una baja en el pH, provoca la fusión de la proteína G con la membrana del endosoma, lo que produce un cambio conformacional en la proteína G y la posterior liberación de la ribonucleoproteína . Una vez que este complejo se encuentra en el citoplasma, se inicia la transcripción primaria de los genes virales de forma monocistrónica y siguiendo un orden decreciente de acuerdo con su concentración molar (N>P>M>G>L), para posteriormente ser traducidos en el ribosoma. Este proceso, es totalmente dependiente de la caperuza en los RNA mensajeros virales, la cual se une al factor de iniciación de la traducción eIF4E [33].

Una vez traducidas las proteínas virales, en el caso de las proteínas M y G, estas son trasportadas por proteínas chaperonas en la célula a la membrana plasmática (proteína M) y al retículo endoplásmico y aparato de Golgi (proteína G). En estos organelos, la proteína G es glicosilada y posteriormente secretada a la membrana plasmática celular externa [27,34].

El proceso de replicación inicia una vez que existe un incremento en la concentración de proteínas virales en la célula, especialmente de la proteína N. Los niveles de esta proteína parecen ser el principal mediador para que la proteína L ignore las señales de paro en las regiones intergénicas y comience a replicar el genoma completo. A su vez, este cambio funcional es promovido por cambios en la estequiometria en el complejo proteico L-P. Durante este proceso de replicación, se forman antigenomas virales (RNA vírico en sentido positivo: 5'-N-P-M-G-L-3'), los cuales servirán como molde de replicación para la formación de genomas virales y posteriormente formarán parte de nuevos complejos de replicación (ribonucleoproteínas), e iniciar así un proceso de transcripción secundaria [27, 34].

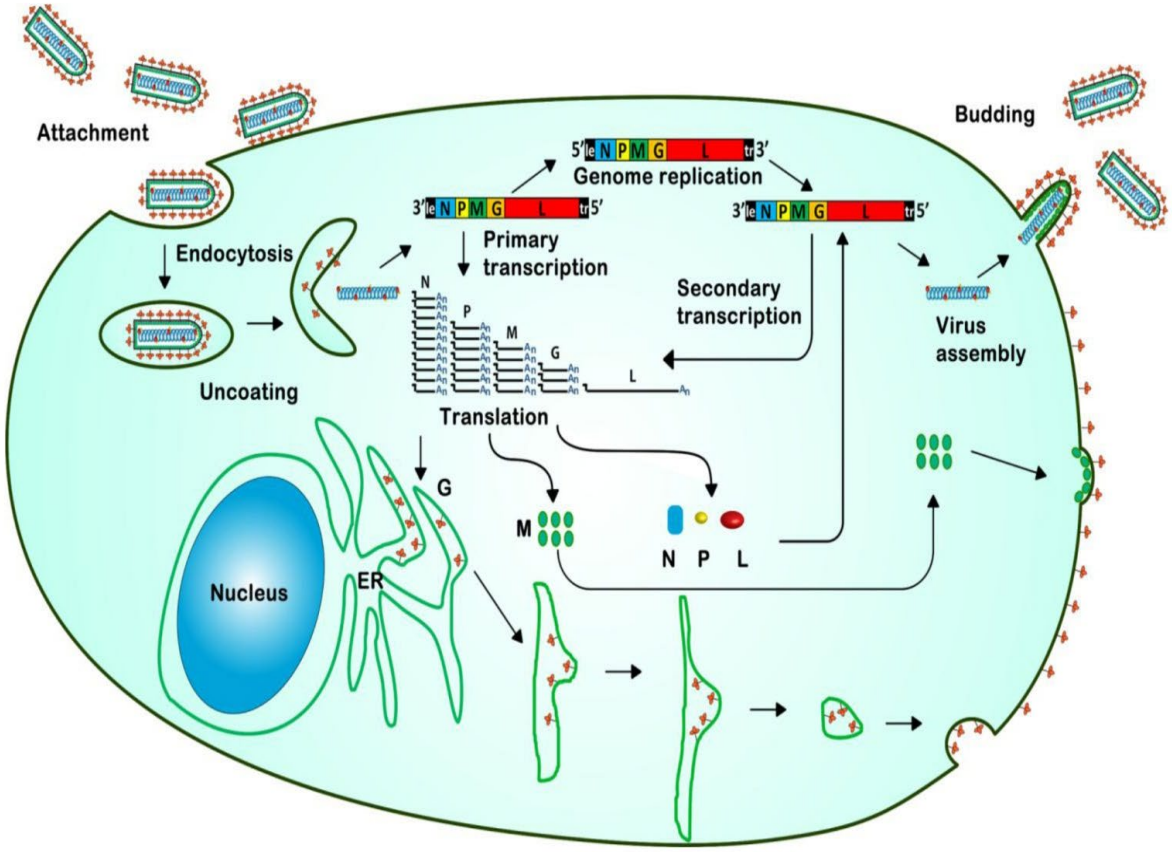

Figura 5. Ciclo de replicación del virus de estomatitis vesicular.

Fuente: Li, J. and Zhang, Y. InTech. 2012, doi: 10.5772/54598 [34]

Finalmente, los nuevos complejos de ribonucleoproteínas son trasportados a la membrana interna mediante microtúbulos [35], para que, junto a la proteína M se inicie el ensamblaje de las nuevas partículas virales, las cuales, incluirán la membrana plasmática del hospedador para formar la envoltura lipídica. En este punto cuando la partícula viral esta lista para salir de la célula, la proteína G se une a la membrana lipídica para completar los nuevos viriones maduros [34].

Es interesante mencionar que, la elevada propensión de la proteína L a competer errores durante la replicación, contribuye al aumento de la diversidad genética de este virus y a la formación de poblaciones de cuasiespecies, las cuales facilitan la rápida adaptación del VSV a diversos ambientes [36]. Estudios evolutivos llevados a cabo *in vitro* en poblaciones del VEV alternando pasajes entre células de mamíferos e insectos, muestran que la habilidad del virus para adaptarse a crecer en ambos hospedadores no limita la acumulación de mutaciones en el genoma viral durante la replicación en cada uno de los hospedadores [37].

1.4 MECANISMOS DEL VSV PARA EVADIR LA RESPUESTA INMUNE INNATA

La respuesta inmune innata desencadenada por el interferón tipo I (IFNI), juega un papel esencial en el control de la infección por el VSV [29]. Durante la infección, diferentes sensores celulares pueden alertar a la célula sobre la presencia del VSV. En este sentido, receptores tipo toll (TLR) pueden detectar a la proteína G en la superficie celular (TLR 4 y TLR 13), y al RNA viral dentro del endosoma celular (TLR 7 y TLR 8) (figura 6) [38, 39, 40].

Asimismo, los sensores citoplasmáticos retinoic acid-inducible gene I (RIG-I) y melanoma differentiation-associated protein 5 (MDA5), han mostrado su habilidad para detectar el RNA viral e intermediarios replicativos de doble cadena del VSV, respectivamente [41, 42]. Posteriormente, una vez que el VSV es detectado por alguno de estos receptores, se inician diferentes cascadas de señalización, orquestadas por el factor nuclear NF-kb y los factores reguladores de interferón 3 (IRF3) y 7 (IRF7), los cuales desencadenan la transcripción del gen del interferón beta (IFN β) [29].

En este punto, el VSV es capaz de impedir la traducción de RNA mensajeros en el hospedador, afectando así, la producción de IFN β, y por lo tanto evitando la acción autocrina y paracrina de esta citoquina, previniendo la activación de la vía de señalización Janus cinasa-STAT (JAK-STAT), afectando la expresión del grupo de genes estimulados por el interferón (ISG) [43]. Entre las proteínas con actividad antiviral contra el VSV es posible mencionar: *Interferon Induced Protein With Tetratricopeptide Repeats 2* (IFIT2), *MX Dynamin Like GTPase 1* (MX1), *2'-5'-Oligoadenylate Synthetase 1*(OAS1), *ribonuclease 4 or 2'-5' oligoadenylate synthetase-dependent ribonucleas* (RNAseL), *Interferon-stimulated gene 15* (ISG15), *Interferon-induced transmembrane protein 3* (IFITM3), y *bone marrow stromal antigen 2* (Terherin) [29].

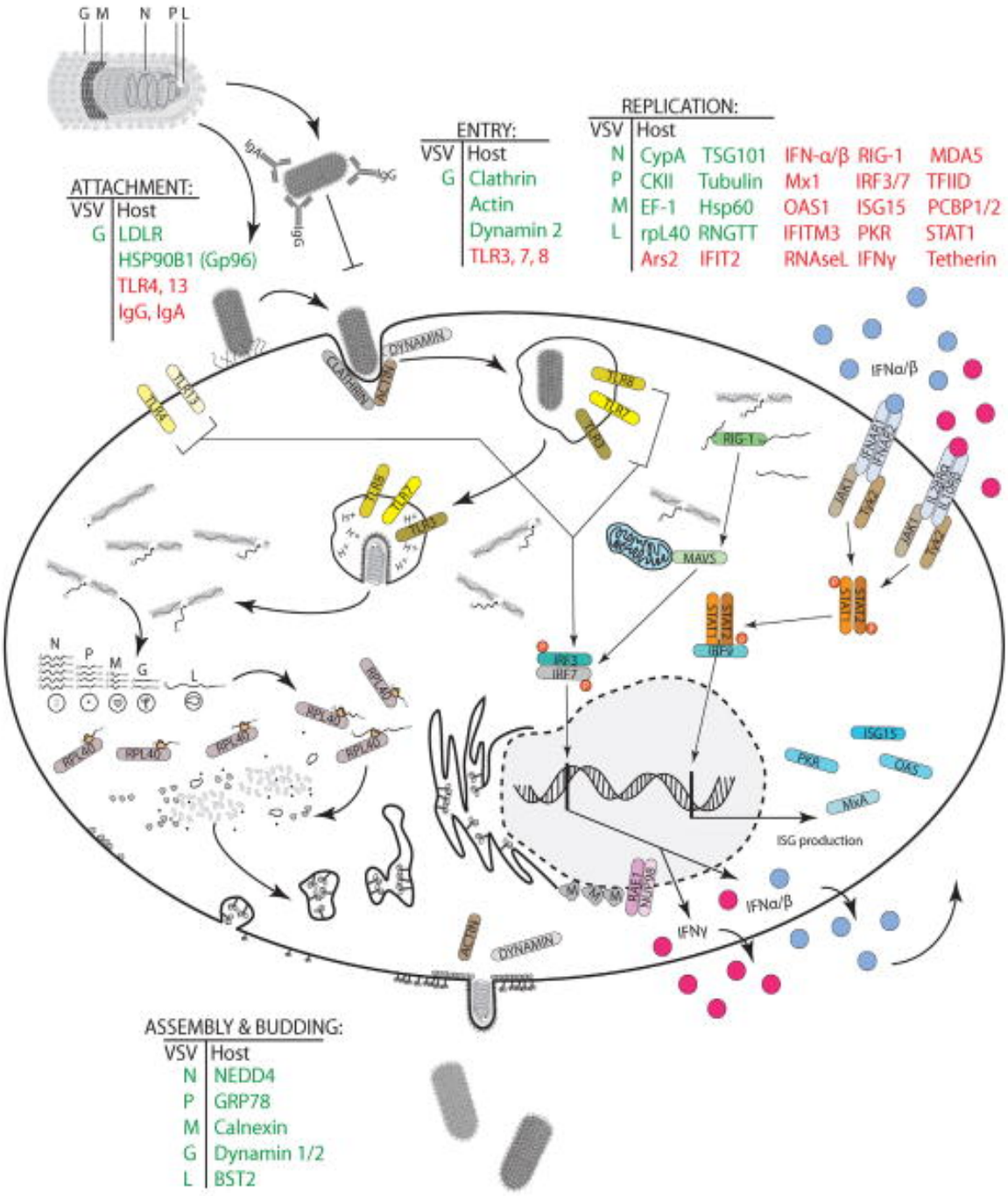

Figura 6. Componentes celulares asociados con el tropismo del VSV. Las proteínas virales o del hospedador asociadas con diferentes pasos del ciclo replicativo del VSV se muestran en verde, mientras en que en rojo se muestran proteínas asociadas con la respuesta viral del hospedador.

Fuente: Hastie E, et al. Virus Res. 2013;176(1-2):16-32 [29].

La habilidad del VSV para afectar la respuesta antiviral del hospedador, está dada principalmente por a la proteína M, una de las proteínas más estudiadas del VSV [29, 44, 45, 46]. Para este fin, parte de la proteína M producida durante la infección, se sitúa en el núcleo celular, produciendo la interrupción de las actividades celulares ligadas con la trascripción, traducción y la exportación nuclear de mensajeros de ARN al citoplasma, afectando así, la producción de múltiples proteínas celulares, entre ellas las asociadas con la respuesta inmune [29, 44, 45,46].

En este sentido, la propiedad de la proteína M para afectar la trascripción en el hospedador, esta mediada por la inactivación de la proteína de unión a TATA (TBP), una subunidad del factor de trascripción basal (TFIID), el cual se une a la región promotora (caja TATA) para iniciar el reclutamiento de los diferentes factores celulares requeridos por la RNA polimerasa II (RNAP II) para iniciar la trascripción (Figura 7) [47].

Por otro lado, en el caso del proceso de traducción, investigaciones sugieren que, durante la infección con el VSV, ocurren dos periodos de inhibición de la traducción celular. El primer periodo se presenta temprano durante la infección (3-6 horas post infección), donde el VSV bloquea selectivamente la producción de proteínas del hospedador, por medio de la desfosforilación de la proteína 4E-BP1 [48]. Este proceso altera el complejo de traducción eIF4F, evitando así, que se lleve a cabo la traducción dependiente de caperuza [49].

Alternativamente para promover la traducción de los RNA mensajeros virales, el VSV utiliza una vía de traducción alternativa promovida por la proteína RpL40, la cual se encuentra presente en la subunidad mayor del ribosoma y es potencialmente usada por RNA mensajeros específicos en la célula [50].

Otro mecanismo por el cual el VSV afecta el proceso de traducción, consiste en interrumpir la exportación de RNA mensajeros del núcleo al citoplasma. Esta función esta mediada por la habilidad de la proteína M para asociarse con la proteína del complejo del poro nuclear NUP-98 (involucrada en el transporte bidireccional entre el núcleo y el citoplasma) y con la proteína de exportación de mensajeros de ARN RAE-1. Esta función de la proteína M está controlada por sus residuos 51 al 59 [51, 52].

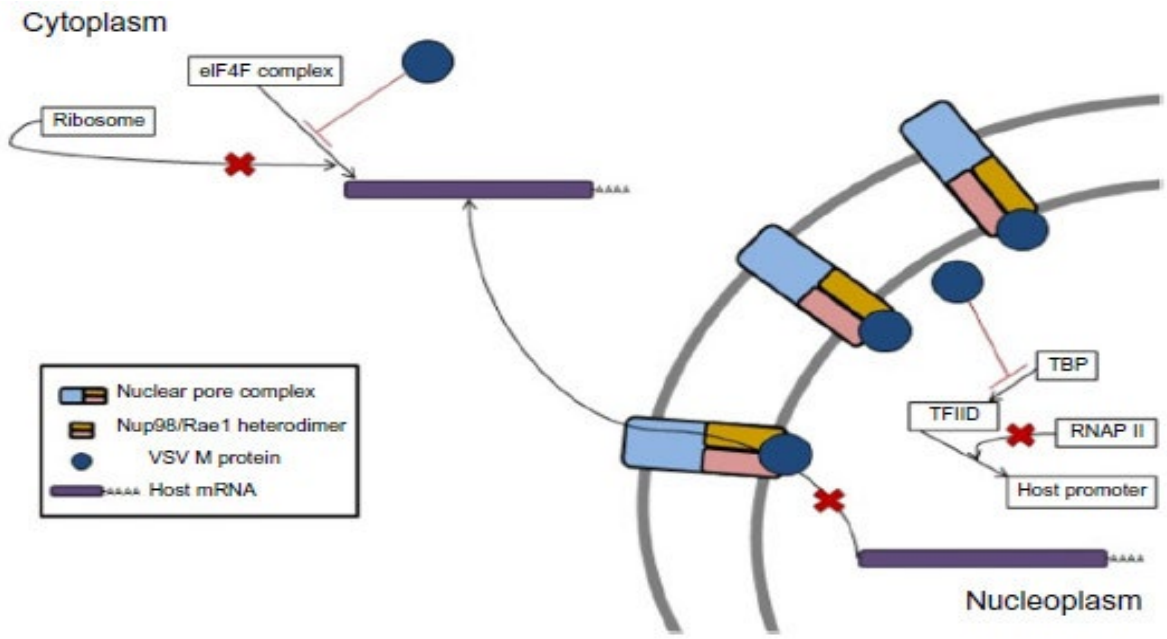

Figura 7. Mecanismos de acción de la proteína M del VSV asociados con la interrupción de la expresión génica en hospedador.

Fuente: Simovic B, et al. Oncolytic Virother. 2015;4:157-67 [52]

Posteriormente, el segundo proceso en la inhibición de la traducción ocurre durante una fase tardía de la infección, afectando en esta ocasión tanto la producción de proteínas del hospedador como del virus. Este proceso esta mediado por la alteración del factor de iniciación eIF2, mediante la fosforilación de la subunidad eIF2α y la consecuente terminación del proceso de traducción. Sin embargo, este proceso parece estar mediado por la proteína quinasa R (PKR), la cual es activada por la detección de intermediarios replicativos del VSV [48].

Por otro lado, algunos tipos celulares inmunes incluyendo las células dendríticas, son capaces de mantenerse funcionales durante la infección con el VSV [53]. En otros casos como los macrófagos, estas células permiten la replicación del VSV con la finalidad de autoestimular la producción de citoquinas, y promover así la estimulación de otros tipos celulares, como las células *natural killer* (NK)[54].

1.5 CICLO INFECCIOSO DEL VSV EN LA NATURALEZA

El VSV es considerado como un arbovirus, el cual comparte su ciclo infeccioso en la naturaleza entre invertebrados y vertebrados [55]. Típicamente, en las zonas endémicas, los arbovirus son mantenidos en un huésped vertebrado (típicamente de origen murino o aviar), los cuales sirven como fuente de infección para los invertebrados hematófagos. En estos huéspedes, el virus se replica, y es ocasionalmente trasmitido por los mosquitos durante su rutina de alimentación a los humanos y animales domésticos, que son considerados los huéspedes finales de este ciclo [56].

Sin embargo, en algunas ocasiones, algunas cepas epizoóticas como las del virus de encefalitis equina venezolana, presentan mutaciones en su genoma, las cuales son capaces de incrementar la virulencia, e inducir una fase de viremia en el huésped final, promoviendo así, la exacerbada trasmisión viral observada durante las epidemias. En otras ocasiones, estas mutaciones permiten aumentar la capacidad viral para replicarse en vectores invertebrados ajenos a las zonas endémicas, permitiendo así su diseminación durante las epizootias [57].

En el caso del ciclo infeccioso del VSV en la naturaleza, muchos aspectos permanecen desconocidos, debido a las múltiples variables asociadas con la epidemiología de esta enfermedad. Esto incluye: habilidad para infectar un amplio rango de animales vertebrados, diferentes rutas de trasmisión, así como un número elevado de vectores invertebrados capaces de trasmitir al virus de manera biológica o mecánica [1, 3].

El VSV es mantenido en nichos ecológicos estables en Centro y Sur América, donde anualmente casos clínicos son reportados en animales domésticos [58]. Sin embargo, ocasionalmente y favorecido por condiciones epidemiológicas desconocidas, el VSV es capaz de escapar de los nichos endémicos, y producir epizootias de manera esporádica, tal y como sucede en los Estados Unidos con los virus provenientes de las zonas endémicas de México [4].

Investigaciones de campo realizadas durante epizootias, muestran que, las rutas de transmisión están claramente vinculadas con distintos vectores invertebrados del orden de los dípteros y las familias: *Ceratopogonidae,* Culicidae y *Psychodidae* [3, 55]. En este sentido, uno

de los grandes enigmas en la epidemiologia del VSV, es en referencia al huésped vertebrado capaz de producir una fase de viremia sostenida durante la infección con este virus, y el cual pueda servir como amplificador viral y como fuente de virus para los insectos [1].

Basado en la detección de anticuerpos neutralizantes en diferentes especies silvestres, recientemente ha sido propuesto un modelo de transmisión, donde venados, murciélagos, ratas y ratones entre otros animales silvestres, podrían estar participando en el mantenimiento del VSV en las zonas endémicas junto a diferentes vectores invertebrados (Figura 8) [3]. Asimismo, le patrón de correlación positiva entre múltiples variables incluyendo: tiempo, espacio, aislamiento viral de insectos, y la presentación de casos clínicos en zonas endémicas y epidémicas, sugieren claramente que los insectos juegan un papel relevante no solo en la introducción de la enfermedad en los hatos, sino también en la diseminación del VSV durante eventos epizoóticos. Una vez en el hato, el virus puede ser transmitido por contacto directo, o mecánicamente [1,3].

Por otro lado, experimentalmente no ha sido posible demostrar la existencia de una fase de viremia en animales domésticos y silvestres infectados experimentalmente con VSV [59, 60, 61, 62, 63, 64, 65, 66]. En este contexto, se han propuesto diferentes mecanismos de transmisión entre insectos para mantener la circulación del VSV en esta especie. Evidencia experimental en moscas negras (*Simulium vittatum*) infectadas con el VSNJV, indica que, en ausencia de un huésped viremico, estas son capaces de infectar a otras moscas durante las rutinas de alimentación de estos insectos en roedores no infectados, mediante un mecanismo denominado *co-feeding* [67]. Igualmente, se ha demostrado la habilidad del VSIDV para ser transmitido en insectos de manera transovárica [68].

Finalmente, en ausencia de un huésped vertebrado capaz de desarrollar una fase de viremia durante la infección con VSV, evidencia experimental sugiere que, las lesiones vesiculares podrían ser una fuente importante para la infección de insectos [69]. Esto ha llevado a proponer una hipótesis sugiriendo que las cepas del VSV con una capacidad mayor para producir cuadros clínicos más severos, podrían tener una mayor habilidad para diseminarse durante las epizootias [7].

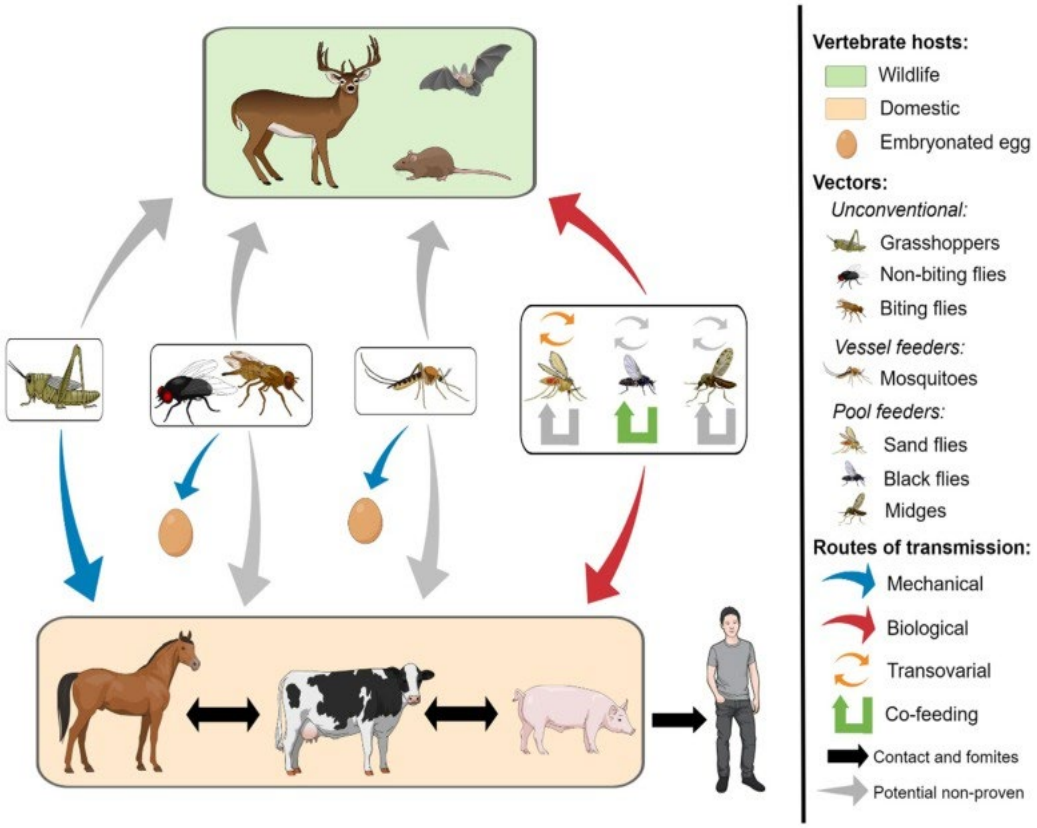

Figura 8. Ciclo infeccioso en la naturaleza propuesto para el VSV.

Fuente: Rozo-Lopez P, et al. Insects. 2018;9(4).

CAPITULO 2

JUSTIFICACIÓN:

El VSNJV ha sido responsable de diversas epizootias durante las últimas tres décadas, afectando diversas especies de interés zootécnico, en las zonas centro norte de México y el sur de los Estados Unidos [1, 4, 12]. Actualmente es sabido que los linajes responsables de estas epizootias reemergen de manera cíclica a partir de progenitores endémicos establecidos en la zona sur de México[4].

Sin embargo, las características biológicas distintivas entre los linajes epidémicos y endémicos del VSNJV son totalmente desconocidas. Al ser VSNJV considerado como un arbovirus [1,4], estas características podrían estar ligadas con ventajas adaptativas para replicarse de manera más eficiente en huéspedes vertebrados, invertebrados, o posiblemente en ambos, tal y como ha sido descrito en otros arbovirus como el virus de encefalitis equina venezolana [15].

El comenzar a elucidar las características biológicas de los linajes epidémicos, contribuirá de manera importante a entender los mecanismos ligados a la reemergencia del VSNJV.

CAPITULO 3

HIPÓTESIS:

Las cepas epidémicas del VSNJV podrían representar un fenotipo más virulento para el cerdo, que su ancestro endémico más cercano.

CAPITULO 4

4.1 OBJETIVO GENERAL

Determinar características biológicas distintivas entre las cepas epidémicas y endémicas del VSNJV, durante la infección en el cerdo, un huésped natural de este virus[1].

4.2 OBJETIVOS ESPECÍFICOS

I. Realizar la secuenciación completa de la cepa epidémica del VSNJV NJ0612NME6 (linaje 1.1) y su pariente endémico mas cercano la cepa NJ0806VCB, con la finalidad de determinar las diferencias nucleotídicas entre ambas cepas.

II. Realizar estudios comparativos de la patogenicidad de las cepas NJ0612NME6 y NJ0806VCB, con la finalidad de evaluar diferencias en la virulencia en el cerdo.

III. Desarrollar una clona de cDNA utilizando el genoma de la cepa NJ0612NME6 utilizando el método de recombinación homologa[22], con la finalidad de manipular genéticamente a esta cepa.

IV. Evaluar la patogenia del virus recombinante producido a partir de la clona de cDNA de la cepa NJ0612NME6, con la finalidad de verificar si los niveles de virulencia de este virus son comparables con los producidos por la cepa parental en el cerdo.

V. Desarrollar por mutagénesis dirigida la mutante M51R, con la finalidad de realizar experimentos de patogenicidad en cerdos y obtener más evidencia sobre los factores asociados al la virulencia de VSNJV en el cerdo.

CAPÍTULO 5

Secuencias genómicas completas de dos virus de estomatitis vesicular New Jersey, los cuales representan a la cepa epidémica NJ0612NME6 (linaje 1.1) que afectó a los Estados Unidos en el 2012 y a su ancestro endémico más cercano la cepa NJ0806VCB (linaje 1.2) que circuló en el sur de México en el 2006.

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VIRUSES

Complete Genome Sequences of Two Vesicular Stomatitis New Jersey Viruses Representing the 2012 U.S. Epidemic Strain and Its Closest Relative Endemic Strain from Southern Mexico

Lauro Velazquez-Salinas,[a,b] Steven J. Pauszek,[a] Antonio Verdugo-Rodriguez,[b] Luis L. Rodriguez[a]

[a]Foreign Animal Disease Research Unit, Plum Island Animal Disease Center, ARS, USDA, Orient Point, New York, USA

[b]College of Veterinary Medicine and Animal Science, National Autonomous University of Mexico, Mexico City, Mexico

ABSTRACT We report here the complete genome sequences of two vesicular stomatitis New Jersey virus (VSNJV) field strains isolated from epithelial lesions from naturally infected animals in Mexico and the United States. The close phylogenetic relationship of these isolates makes them an ideal model for assessing potential genetic factors linked with the emergence of VSNJV in the United States.

Vesicular stomatitis virus (VSV) affects horses, pigs, and cattle throughout the Americas, causing a disease known as vesicular stomatitis (VS), which results in vesicular epithelial lesions. In cattle and swine, the clinical signs resemble those caused by foot-and-mouth disease virus (1).

VSV is the prototype virus for the *Rhabdoviridae* family and the genus *Vesiculovirus*, for which two main serotypes have been described, vesicular stomatitis New Jersey virus (VSNJV) and vesicular stomatitis Indiana virus (VSIV) (2). VSV has an ~11-kb negative-sense single-strand genome which codes for five structural proteins (the nucleoprotein [N], phosphoprotein [P], matrix protein [M], glycoprotein [G], and polymerase [L]) and two nonstructural proteins (C and C′) that are encoded in a second open reading frame within the P gene (2, 3).

Important gaps remain in our understanding of the epidemiological and virulence factors mediating cyclical disease emergence in the United States. Previous studies have shown that there is a close ancestral genetic relationship between viral strains causing outbreaks in the United States and viruses circulating in regions of endemicity in southern Mexico (4, 5). However, the genetic determinants of virulence associated with the emergence of epidemic strains remains unclear. In this context, it is essential to have well-characterized viral strains and reliable animal models to associate *in vivo* phenotype with genotype characteristics associated with disease emergence.

In this announcement, we report two full-length genome sequences of VSNJV, one isolated from southern Mexico in 2006 (NJ0806VCB) and the other isolated from the southwestern United States in 2012 (NJ0612NME6).

Viral strain NJ0806VCB was isolated in 2006 from a cow naturally infected in the state of Veracruz, Mexico, where the virus is endemic, and viral strain NJ0612NME6 was recovered from epithelial lesions collected from a horse in New Mexico during an outbreak of VSNJV in 2012. Phylogenetic analysis indicated that viral strain NJ0612NME6 belongs to the epidemic lineage 1.1 and viral strain NJ0806VCB belongs to the endemic lineage 1.2, the latest common endemic ancestor of lineage 1.1 responsible for the outbreaks in the United States in 2012 (5).

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Address correspondence to Luis L. Rodriguez, luis.rodriguez@ars.usda.gov.

Viral sequences were obtained as previously described (6). Viral RNA was extracted from cell culture (one passage in Vero cells) supernatants using the RNeasy minikit (Qiagen). The full-length genome sequence of each viral isolate was amplified in 19 overlapping fragments (~600 to 700 bp) by reverse transcription-PCR (RT-PCR) using a specific set of primers. RT-PCRs were conducted using the SuperScript III one-step RT-PCR Platinum *Taq* high-fidelity kit (Invitrogen). RT-PCR products were analyzed on a 1.5% agarose gel stained with SYBR safe DNA gel stain (Thermo Fisher) and visualized by UV transillumination and then purified using QIAquick PCR purification columns (Qiagen) and sequenced using the dideoxy termination method with a Big Dye terminator (Life Technologies). To assemble consensus sequences of each virus, chromatograms were analyzed using Sequencher v4.8 (Gene Codes, Ann Arbor, MI, USA). The genomic termini were obtained as previously described (7).

These two viral sequences are useful for identifying genetic determinants and possibly distinctive biological characteristics between endemic and epidemic VSNJV strains as well as for the construction of infectious clones to study genetic factors linked to virulence.

Accession number(s). The complete genome sequences of NJ0806VCB and NJ0612NME6 described herein have been deposited in GenBank under the accession no. MG552608 and MG552609, respectively.

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This work was part of a collaboration between the laboratories of the Mexico-United States Commission for the Prevention of Foot and Mouth Disease and Other Exotic Animal Diseases (CPA) in Mexico and the Plum Island Animal Disease Center in the United States.

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CAPÍTULO 6

El incremento en la virulencia de la cepa epidémica NJ0612NME6 del virus de estomatitis vesicular, está asociada con la capacidad de esta cepa para interferir en la respuesta inmune innata en el cerdo.

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Increased Virulence of an Epidemic Strain of Vesicular Stomatitis Virus Is Associated With Interference of the Innate Response in Pigs

*Lauro Velazquez-Salinas[1,2,3], Steven J. Pauszek[1], Carolina Stenfeldt[1,4], Emily S. O'Hearn[5], Juan M. Pacheco[1], Manuel V. Borca[1], Antonio Verdugo-Rodriguez[2], Jonathan Arzt[1] and Luis L. Rodriguez[1]**

[1] Foreign Animal Disease Research Unit, Plum Island Animal Disease Center, United States Department of Agriculture–Agricultural Research Service, Greenport, NY, United States, [2] College of Veterinary Medicine and Animal Science, National Autonomous University of Mexico, Mexico City, Mexico, [3] Plum Island Animal Disease Center Research Participation Program, Oak Ridge Institute for Science and Education, Oak Ridge, TN, United States, [4] Department of Veterinary Population Medicine, University of Minnesota, St. Paul, MN, United States, [5] Foreign Animal Disease Diagnostic Laboratory, Veterinary Services, Animal and Plant Health Inspection Service, United States Department of Agriculture, Plum Island, NY, United States

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***Correspondence:**
Luis L. Rodriguez
luis.rodriguez@ars.usda.gov

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Vesicular stomatitis virus (VSV) causes sporadic outbreaks of vesicular disease in the southwestern United States. The intrinsic characteristics of epidemic strains associated with these outbreaks are poorly understood. In this study, we report the distinctive genomic and biological characteristics of an epidemic (NJ0612NME6) strain of VSV compared with an endemic (NJ0806VCB) strain. Genomic comparisons between the two strains revealed a total of 111 nucleotide differences (23 non-synonymous) with potentially relevant replacements located in the P, G, and L proteins. When tested in experimentally infected pigs, a natural host of VSV, the epidemic strain caused higher fever and an increased number of vesicular lesions compared to pigs infected with the endemic strain. Pigs infected with the epidemic strain showed decreased systemic antiviral activity (type I – IFN), lower antibody levels, higher levels of interleukin 6, and lower levels of tumor necrosis factor during the acute phase of disease compared to pigs infected with the endemic strain. Furthermore, we document the existence of an RNAemia phase in pigs experimentally infected with VSV and explored the cause for the lack of recovery of infectious virus from blood. Finally, the epidemic strain was shown to be more efficient in down-regulating transcription of IRF-7 in primary porcine macrophages. Collectively, the data shows that the epidemic strain of VSV we tested has an enhanced ability to modulate the innate immune response of the vertebrate host. Further studies are needed to examine other epidemic strains and what contributions a phenotype of increased virulence might have on the transmission of VSV during epizootics.

Keywords: vesicular stomatitis, parthenogenesis, virulence, interferon, immune response, IL-6, TNF, epidemics

INTRODUCTION

Vesicular stomatitis (VS) is caused by the arthropod-borne vesicular stomatitis virus (VSV, family Rhabdoviridae, genus *Vesiculovirus*). The non-segmented RNA viral genome of VSV encodes five structural proteins: nucleocapsid (N), phosphoprotein (P), matrix (M), glycoprotein (G), and the large RNA-dependent RNA polymerase (L) (Wagner and Rose, 1996; Dietzgen, 2012) along with two non-structural proteins (C and C′) of undetermined function encoded from overlapping reading frames in the P gene (Spiropoulou and Nichol, 1993). VSV causes most of the cases of vesicular disease reported in livestock (Rodriguez, 2002; Mead et al., 2009). Because its clinical signs in cattle and pigs are indistinguishable from those of foot-and-mouth disease (FMD), VS reports result in costly animal quarantines and trade embargoes (Velazquez-Salinas et al., 2014). Unlike FMD, VS also occurs in horses, resulting in animal movement restrictions that cause disruption to trade shows (Bridges et al., 1997; Timoney, 2016).

Taxonomically, VSV is classified into two distinct serotypes: New Jersey (VSNJV) and Indiana (VSIV) (Rodriguez, 2002; Dietzgen, 2012). Both of these serotypes have been reported from as far south as Bolivia to as far north as the United States. VSV is considered endemic from southern Mexico throughout Central America to northern South America, where multiple genetic lineages co-circulate each year. VS occurs less frequently in Mexico's central and northern states where its occurrence is usually associated with single genetic lineages (Arroyo et al., 2011). Outbreaks of VS in the United States have historically occurred every 8–10 years with outbreaks often extending for 1–3 years after the first occurrence. Outbreak cycles usually initiate in the border states of Arizona, New Mexico, or Texas and spread as far north as Wyoming (Rainwater-Lovett et al., 2007; Perez et al., 2010). Phylogeographic studies indicate that epidemic VSNJV strains affecting the United States are monophyletic lineages emerging from enzootic progenitors circulating in southern Mexico (Rodriguez et al., 2000; Rainwater-Lovett et al., 2007; Velazquez-Salinas et al., 2014).

Recently, we provided a detailed description of the emergence and progressive northward migration of a particular VSNJV lineage, termed 1.1, which spread through central and northern Mexico between 2006 and 2009 and into the southern United States in 2012. Phylogenetic characterization based on the hypervariable region of the P gene strongly suggested that the latest common endemic ancestor of lineage 1.1 was lineage 1.2, a group of viruses confined in the endemic states of Veracruz and Tabasco (Velazquez-Salinas et al., 2014). However, the inherent molecular and biological differences between epidemic and endemic VSV strains remain poorly understood.

The aim of the current study was to examine the molecular and biological characteristics of an epidemic emerging strain of VSV (NJ0612NME6) compared to that of a closely (genetically) related endemic strain (NJ0806VCB). We conducted a comprehensive genomic analysis to determine relevant nucleotide and amino acid substitutions associated with the genetic divergence between the lineages. Furthermore, using a well-established model for pathogenesis studies in domestic pigs (a natural, vertebrate host of VSV) we sought to determine the biological differences between these two lineages. The findings of this study are discussed regarding mechanisms of viral pathogenesis associated with epidemic lineages of VSV, as well as their potential impact during epizootics of VSV.

MATERIALS AND METHODS

Viral Strains

Two VSNJV strains, each representing one of the two genetic lineages, were used for this study. Viral strain NJ0806VCB, the lineage 1.2 representative virus, was isolated from a naturally infected bovine in Veracruz in 2006, where VSNJV occurs endemically (Arroyo et al., 2011). Viral strain NJ0612NME6, the lineage 1.1 representative virus, was isolated from a naturally infected equine in New Mexico during the 2012 VSNJV outbreak in the United States. Both viruses were obtained as first passage in Vero cells and were propagated once at an MOI of 0.01 $TCID_{50}$ in the same cell line to produce high titer viral stocks that were stored at −70°C until usage. Viral strain VSIV-IFN-βb-NIS, which constitutively expresses human interferon beta (Naik and Russell, 2009) was used as a positive control for *in vitro* cytokine expression experiments and was kindly provided by Dr. Shruthi Naik.

Cell Lines

The Vero (Vervet Monkey Kidney Epithelial cells) and BHK-21 (baby hamster kidney cells) cell lines were obtained from ATCC (ATCC catalog numbers CCL-81 and CCL-10, respectively). Primary fetal swine kidney cell cultures were obtained from the Foreign Animal Disease Diagnostic Laboratory (FADDL) at the Plum Island Animal Disease Center (PIADC), Greenport, NY, United States. Porcine peripheral blood was used to derive primary swine macrophage cell cultures as previously described (Zsak et al., 1996).

Phylogenetic Analysis

Full-length genomic sequences of NJ0806VCB (accession #MG552608) and NJ0612NME6 (accession #MG552609) were obtained by the Sanger method as previously reported (Velazquez-Salinas et al., 2018). The evolutionary history of lineage 1.1 as well as its relationship with previously determined VSNJV strains affecting the United States and Mexico (Velazquez-Salinas et al., 2017a) was determined by phylogenetic analysis using relevant whole genome sequences available in GenBank. The genetic relationships were inferred using the MEGA 7 software package (Kumar et al., 2016) under the Maximum Likelihood optimality criterion with the General Time Reversible model and allowing some sites to be evolutionary invariable (Nei and Kumar, 2000). To evaluate the robustness of the tree, we applied a bootstrap analysis with 1,000 replicates. Additionally, synonymous (dS) and non-synonymous (dN) pairwise distance analyses were carried out using the program Sequence Distances in the SSE software version 1.2 (Simmonds, 2012).

Amino Acid Substitution Analysis

The probability of a biologically meaningful amino-acid replacement occurring in the protein alignment was assessed using the Blocks Substitution Matrix (BLOSUM80). Positive scores imply a favored change; a zero score indicates a neutral change, and negative scores suggest a disfavored change (Henikoff and Henikoff, 1992; Betts and Russell, 2003).

In vitro Growth Characterization

In vitro growth characteristics of NJ0612NME6 and NJ0806VCB were evaluated using multistep growth curves. Primary fetal swine kidney cell cultures and primary swine macrophage cultures were infected in triplicate at an MOI of 0.01 $TCID_{50}$ per cell. Viruses were absorbed for 1 h (time zero) and samples were collected at 0, 4, 8, 24, 48 h post-infection (hpi). Titrations were conducted in BHK-21 cells as previously described (Martinez et al., 2003). Briefly samples were serially 10-fold diluted and added to the cells (suspension) in octuplicate wells and incubated at 37°C for 72 h. Titers, expressed as $TCID_{50}$/ml, were calculated using the Reed and Muench method (Reed and Muench, 1938).

In addition, the ability of the two viral strains to grow at different temperatures was assessed by titrating high titter stocks of each virus at 32°C, 37°C, and 39°C using preformed monolayers of Vero cells. The ability of each virus to grow at different temperature conditions was quantified as the titer of the virus at either 32°C or 39°C divided by the titer of the virus at 37°C (Thermostability index). Values >1 reflect higher thermostability, values <1 reflect lower thermostability and values equal to 1 no changes in thermostability. Experiments were performed in triplicate.

Transcriptional Regulation of the Immune Response

To evaluate the ability of each virus to regulate the antiviral response during *in vitro* infection, we used a previously described model based on the ability of VSV to disrupt the transcriptional wave of innate response genes in infected cells (Stojdl et al., 2003). Gene expression was determined in infected (MOI of 10 $TCID_{50}$) primary fetal swine kidney cell cultures (FPKC) and primary swine macrophage cultures (SMC). At 5 hpi, total cellular RNA was extracted from mock-infected and infected cells. Steady state levels of mRNA accumulation were determined for 11 swine genes representative of the primary (IFN- β), secondary (IRF7, STAT 2, Mx1, OAS1, and PKR), and tertiary (IFN-α-1, IFN-α-7/11, IFN-α-9, IFN-α-10, and IFN-α-17) transcriptional waves of innate response genes. Analyses were done by quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR) followed by a melting curve analysis as previously described (Borca et al., 2008). A change in the normalized mRNA expression level of a gene in VSV infected cells was deemed significant if it deviated from its respective expression level in mock-infected cells by at least threefold either up or down (Brukman and Enquist, 2006; Borca et al., 2008). As a control, we included a previously described recombinant VSIV engineered to express human IFN beta and the sodium iodide symporter (NIS) as this virus is unable to disrupt the innate immune response in normal cells (Naik and Russell, 2009).

Animal Experiments

Pigs, a natural host of VSV, were used as a model of animal infection. The high susceptibility of this species to VSV has been well established providing an excellent model for pathogenesis studies (Clarke et al., 1996; Howerth et al., 1997; Stallknecht et al., 2001; Martinez et al., 2003).

Studies were conducted under an experimental protocol approved by the institutional animal care and use committee (IACUC protocol #245-05-14R) in a Biosafety Level 3-agricultural (BSL-3Ag) facility at the United States Department of Agriculture's Plum Island Animal Disease Center (PIADC). Two replicate *in vivo* experiments were performed; one with each representative virus. In each of the experiments, eight Yorkshire pigs (8–10 weeks old and 25–30 kg in weight) were housed in the same isolation room. Within the room, pigs were divided into two groups (inoculated and contact) of four pigs each separated by double fencing to avoid direct contact. After a 1 week acclimation period, four pigs were intradermally inoculated in the snout with either NJ0612NME6 or NJ0806VCB as previously described (Martinez et al., 2003). Briefly, an intramuscular injection of a mixture of xylazine, ketamine, and telazol (4, 8, and 3 mg/kg, respectively) was used to sedate the animals. The epidermis of the snout was pricked 20 times using a dual-tip skin test applicator (Duotip-Test: Lincoln Diagnostics, Decatur, III) and 10^7 $TCID_{50}$ of virus in 50 μl of Dulbecco Modified Eagle Medium (DMEM) was placed on the scarified area. The area under the inoculum was re-sensitized by repeating the scarification procedure and the snout was held stationary in an upright, flat position for 3 min to assure sufficient contact time. The inoculated animals were kept separate from the contact group by double fencing through 24 h. The fencing separating the groups was subsequently removed and the contact pigs were allowed to co-mingle with the inoculated group for the duration of the experiment (21 days).

Sampling and Clinical Evaluation

Throughout each of the experiments, sample collection and clinical evaluations were performed following a standardized protocol. Animals were sampled and clinically evaluated daily from days 0 through 10 and again at 14 and 21 days post-infection (dpi). Collected samples included whole blood, serum, oropharyngeal (OP) swabs and nasal swabs. Blood samples were collected from the jugular vein into EDTA-containing tubes (Vacutainer) or into tubes without anticoagulant to obtain serum. OP swabs were collected by directly targeting the tonsil of the soft palate using a large cotton swab while nasal swabs were collected by swiping small cotton swabs within the external nares. Directly after collection, all swabs were immersed in 2 ml minimal essential media containing 25 mM HEPES. The fluid absorbed by the large OP swab was extracted using an additional, brief centrifugation step. After initial processing, samples were immediately stored at −70°C until testing.

The severity and dissemination of the disease was evaluated using a clinical scoring system based on the location and

distribution of the vesicular lesions. In brief, each of the 16 digits with a characteristic lesion contributed two points toward a cumulative score, with a single additional point added for vesicular lesions observed on the snout of the directly inoculated animals or two points for snout lesions on contact animals, two points added for lesions in the lower lip, oral cavity and on carpal/tarsal skin, thus allowing a maximum score of 45 and 46 for direct inoculated and contact pigs, respectively. Rectal temperatures were measured daily before sample collection.

Post-mortem Sample Collection

Four pigs (two inoculated and two contact-exposed) representing the individuals with the highest accumulated clinical score from each of the two experiments, were euthanized for tissue harvest at 21 dpi by deep sedation (see above) followed by exsanguination. Tissues collected included: anterior tongue epithelium (ATONG), tonsil of the soft palate (PTON), nasopharyngeal tonsil (NTON), neck skin (NS), submandibular lymph node (SMLN), liver (LIV), spleen (SPL), snout skin (SNT), gastrohepatic lymph node (GHLN), coronary band-vesicle (CB-V), parotid lymph node (ParLN), and right popliteal lymph node (R-PopLN). Each tissue sample was divided into two 30 mg aliquots that were placed in individual tubes and frozen at −70°C until further processing.

Viral RNA Detection

RNA extraction was carried out using Ambion's MagMax-96 Viral RNA Isolation Kit (Ambion, Austin, TX, United States) on a King Fisher-96 Magnetic Particle Processor (Thermo Scientific Waltham, MA, United States) following a protocol previously described (Arzt et al., 2010). RNA (2.5 μl) was analyzed by real-time RT-PCR (rRT-PCR) targeting the VSNJV nucleocapsid gene (N), following a protocol previously described (Scherer et al., 2007). The only difference compared to the previously published protocol was a single nucleotide change introduced in the forward primer (5′-GCACTTCCTGATGGGAAATCA-3′) to match the sequence of the two viruses used in the current study. Reactions were performed with an ABI 7000 system (Applied Biosystems, Austin, TX, United States). **Supplementary Figure S1** shows the test sensitivity for the detection of these two strains. Cycle threshold values were converted into RNA genome copy numbers per 2.5 μl of RNA by use of standard curves based on analysis of 10-fold dilutions of *in vitro* synthesized VSNJV N RNA.

Virus Isolation

Debris and potential bacterial contamination were cleared from aliquots of macerated tissue samples and fluid from nasal and OP swabs via centrifugation through 0.45 μm Spin-X filter columns (Costar cat. No 8163). The resulting fluids were diluted 1:5 in cell culture medium and 500 μl of each dilution applied to Vero cell monolayers in 24 well plates and observed for cytopathic effect (CPE) for 72 h. All CPE positive samples were confirmed as VSNJV utilizing rRT-PCR and titrated at 37°C in 96-well plates with preformed monolayers of BHK-21 cells. Each sample was tested in eight replicate wells. Titers were calculated as described above.

Neutralizing Activity of Non-immune Porcine Serum

To test the non-specific inhibitory effect of pig serum on VSNJV, non-immune porcine serum (NPS) was collected from five healthy donor pigs, pooled and frozen at −70°C until used. A portion of the NPS was heat inactivated at 56°C for 30 min (NPSH). For neutralization assays, NJ0612NME6 was titrated in 10-fold dilutions in phosphate buffered saline solution (PBS) and 5 μl of each dilution incubated with 100 μl of either NPS or NSPH at 37°C for 1 h (Tesfay et al., 2014). As a control, viral dilutions were incubated with PBS only. After the incubation period, the reaction mixtures were used to infect monolayers of Vero cells and then overlaid with gum tragacanth and incubated at 37°C for 48 h. Finally, plaques were visualized by staining with crystal violet and viral titers were determined and expressed as plaque forming units (PFUs). All virus neutralization assays were conducted in triplicate.

Complement Determination in Pig Serum

The number of complement units in pig serum was determined by a standard hemolytic assay following a previously described protocol (Kabat and Mayer, 1961). Pig sera were twofold diluted in gelatin veronal buffer (GVB) and 100 μl of each dilution was incubated with 200 μl of a sheep erythrocytes suspension (SES) (5% suspension with GVB previously sensitized with a rabbit antibody against sheep erythrocytes, kindly supplied by APHIS-FADDL) at 37°C for 1 h. Controls for the assay included: cell control (mixture of 100 μl of GVB and 200 μl of SES) and total lysis control (mixture of 100 μl of water and 200 μl SES). After incubation, mixtures were centrifuged at 931 × *g* for 10 min at 4°C and supernatants were read at A_{541} in a spectrophotometer. The percentage of hemolysis was calculated as follows: $y = (A_{541}$ sample solution – A_{541} cell control$)/A_{541}$ total lysis control. One hemolytic unit of complement (CH50 unit) was defined as the highest serum dilution that produced 50% of hemolysis.

Antiviral Activity in Serum

Antiviral activity in serum was assessed using the Mx-CAT reporter assay (Fray et al., 2001), as previously described (Perez-Martin et al., 2012; Fernandez-Sainz et al., 2015). Briefly, MDBK-T cells were seeded in 24 well plates and after 24 h, 0.1 ml of either serum or specific amounts of recombinant human IFN-α 2A (1.95–1,000 U/ml) (standard control) were added to the respective wells. After 24 h of incubation at 37°C, cells were lysed and CAT expression was determined using a commercially available ELISA kit (Roche Applied Sciences, Indianapolis, IN, United States). Results were expressed as a unit of antiviral activity per ml. Previous studies have shown that this test measures type-I IFN activity (Fray et al., 2001; Francois et al., 2005).

Detection of Cytokines in Serum of Infected Animals

Levels of total tumor necrosis factor (TNF), a previously characterized cytokine with antiviral activity against VSV

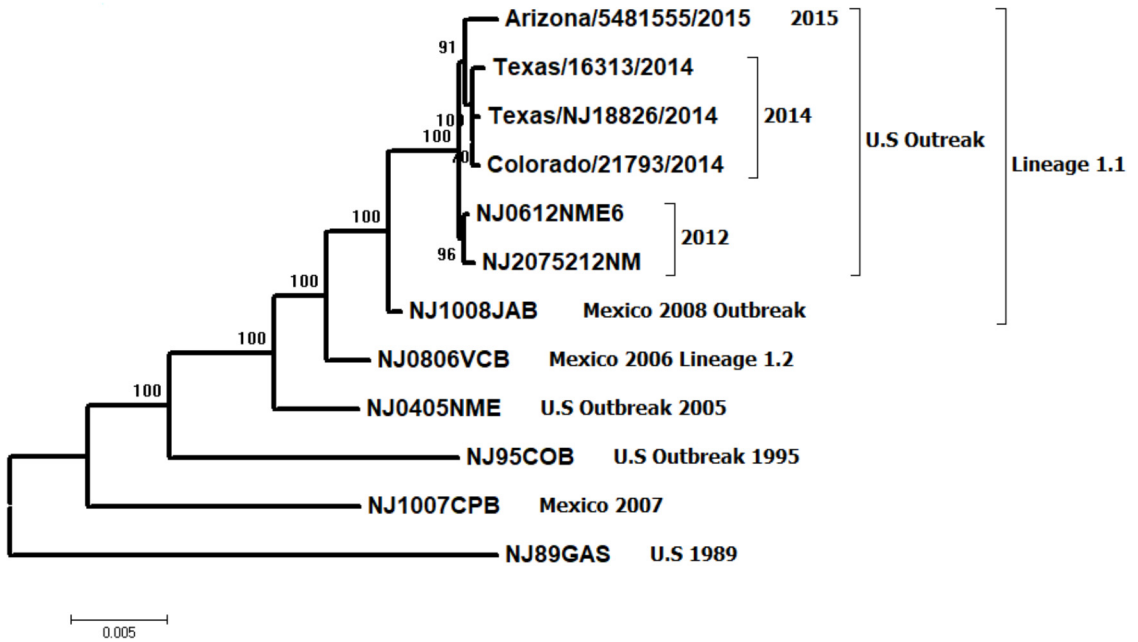

FIGURE 1 | Maximum likelihood phylogenetic analysis of 13 VSNJV from Mexico and the United States, collected between 1989 and 2015 based on the P protein hypervariable region. Notice the relationship between the emergent lineage 1.1 and its closest endemic relative, lineage 1.2 (represented by viral strain NJ0806VCB). These viruses are distinct from previously characterized viral strains isolated during earlier outbreaks in the United States (1995 and 2005) and a viral strain collected in an endemic zone of Mexico during 2007. Numbers above the interior nodes represent bootstrap values obtained after 1,000 replicates.

(Mestan et al., 1988), and interleukin 6 (IL-6), a cytokine associated with immunosuppression activity by affecting the maturation of dendritic cells (Park et al., 2004), were determined by ELISA following the manufacturer's protocol (R&D Systems, Minneapolis, MN, United States).

Serum Neutralization Assay

Neutralizing antibodies against VSNJV were detected as previously described (Flanagan et al., 2001). Briefly, sera were collected and heat inactivated at 56°C for 30 min. Twofold serial dilutions of heat inactivated sera were incubated with 1000 $TCID_{50}$ of VSNJV for 1 h at 37°C in 96-well plates, and after that Vero cells in a concentration of 1×10^6 cells per plate were added in different plates. Plates were incubated at 37°C for 3 days. Serum neutralizing activity was reported as the reciprocal of the highest dilution giving 100% inhibition of CPE.

To estimate the levels of antibodies (IgM and IgG) during the acute phase of the disease, serum samples collected between 0 and 6 dpi were subjected to complement fixation assay (CFA). CFA was performed as previously described (Berninger et al., 2018). Briefly, control and test sera were diluted 1:5 and heat inactivated at 56°C for 30 min. Serial twofold dilutions of each sample were made in 96-well "U" bottom plates, followed by incubation at 37°C for 3 h in presence of complement and VSNJV antigen. A 1.4% solution of sheep red blood cells and hemolysin (rabbit anti-sheep red blood cells) was added and after a 30-min incubation at 37°C, the plates were read for the presence or absence of hemolysis. Endpoint titer was the last dilution showing hemolysis.

Statistical Analysis

Statistical differences ($p < 0.05$) were calculated by the one unpaired *t*-test. This test compares the difference between means with the standard error of the difference, computed by combining the standard errors of the two groups. Each independent variable (inoculation days) was analyzed individually. Normal sample distribution was assessed by the Kolmogorov–Smirnov test. Calculations were performed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla, CA, United States)[1].

RESULTS

Phylogenetic Analysis

To better understand the genetic relationship between viral strains NJ0612NME6 (epidemic lineage 1.1 representative virus from the United States) and NJ0806VCB (endemic lineage 1.2 representative virus from Mexico), a phylogenetic analysis was conducted using their full-length genomic sequences. Additional full-length genomic sequences of lineage 1.1 virus from Mexico (NJ1008JAB) and all available viruses from the United States outbreaks in 1995, 2005, 2012, 2014, and 2015 were included in the analysis. The NJ0612NME6 virus grouped with other lineage 1.1 viruses circulating in Mexico, as far back as 2006 and was ancestral to viruses detected in the United States in 2014 and 2015 (**Figure 1**). The basal branch position of viral

[1]www.graphpad.com

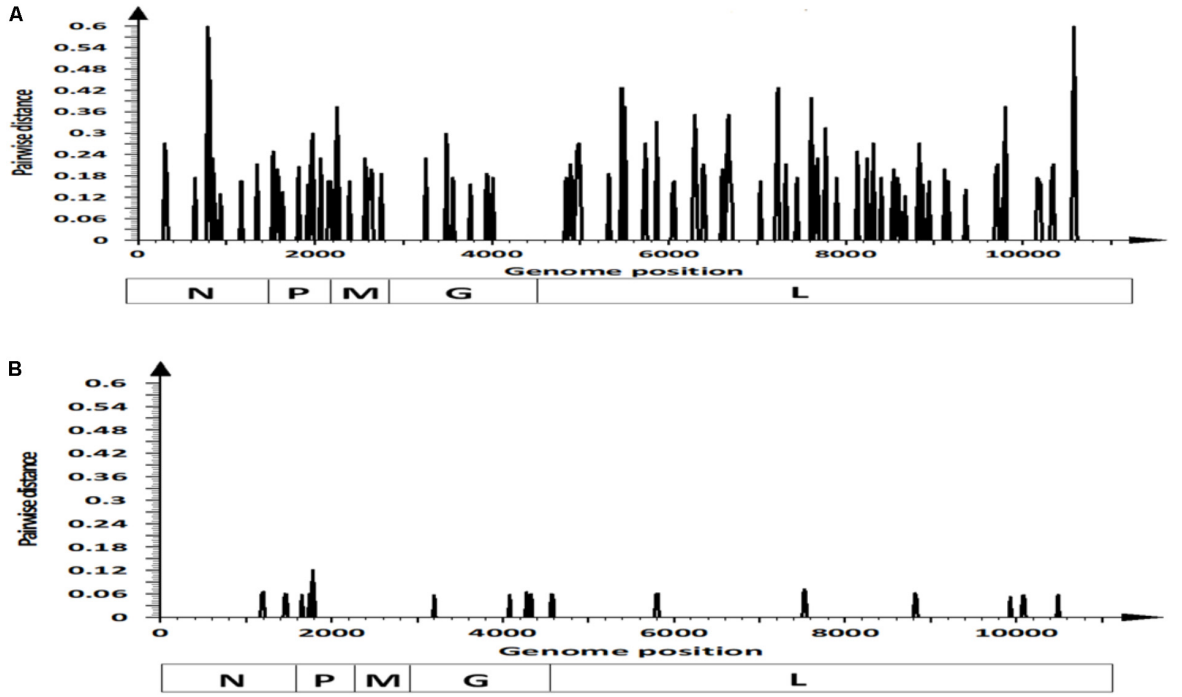

FIGURE 2 | Pairwise distance analysis contrasting differences in: **(A)** synonymous and **(B)** non-synonymous sites within coding regions of viral strains NJ0612NME6 and NJ0806VCB. Bars in the graphics represent pairwise distance comparisons using 20 nt windows.

strain NJ1008JAB suggests that the ancestral source of the viruses circulating in the United States were strains associated with lineage 1.1 previously circulating in central and northern Mexico between 2006 and 2009. After its emergence in the United States in 2012, lineage 1.1 differentiated into sublineages following geographic and temporal distribution but the closest relative to the ancestral NJ1008JAB was NJ0612NME6 (**Figure 1**). Our analysis confirmed that the closest endemic genetic relative to lineage 1.1 was lineage 1.2, which includes NJ0806VCB, and appeared in the phylogenetic tree as a basal branch to lineage 1.1. There was no direct phylogenetic relationship between lineage 1.1 and VSNJV lineages causing previous outbreaks in the United States, clearly indicating that the 2012 emergence of lineage 1.1 in the United States was the result of a new viral incursion from Mexico.

Genomic Comparison

We analyzed nucleotide substitutions between viral strains NJ0612NME6 and NJ0806VCB to identify dS and dN substitutions. There were 111 nucleotide substitutions distributed across the coding regions of these two strains, with a majority of these substitutions (n = 88) being synonymous (**Figures 2A,B**). Overall, most of the 88 synonymous substitutions were located in the L gene (n = 56). Their distribution across previously described functional regions was as follows: N-terminal region (n = 3), conserved region (CR) I (n = 7), CR II (n = 2), CR IV (n = 7), CR V (n = 8), unstructured region (n = 9), CR VI (n = 6), and C-terminal region (n = 6), while eight dS substitutions were located in regions with non-specific functions. The remaining synonymous substitutions (n = 32) were distributed across the remaining genes as follows: M (n = 9), N (n = 8), P (n = 8), and G (n = 7). Only the substitutions in the P gene were located in known functional regions: domain I (n = 3), hypervariable region (n = 2), domain II (n = 1), and domain III (n = 1). Interestingly, of the 88 synonymous substitutions, 60.24% (n = 53) were consistently found in other viral strains associated within lineage 1.1 considered in this study, with 34.1% (n = 30) of them conserved among all viral strains isolated from the recent United States outbreak cycle (i.e., 2012–2015) (**Supplementary Figure S2**).

There were 23 predicted amino acid substitutions between NJ0612NME6 and NJ0806VCB, 19 of which were located in four of the structural proteins (N, P, G, and L) and the remaining four located in the C and C′ proteins encoded in the second reading frame of the phosphoprotein (**Figure 3**). The amino acid changes in the P protein were located in domain I (position 488) and the hypervariable region (positions 549, 580, 592, 595, and 604). Amino acid substitutions in the polymerase (L protein) were distributed across the N- terminal region (positions 1445 and 1526), CR II (position 1934), CR IV (position 2506), CR V (position 2513), unstructured region (position 2941), and C-terminal region (positions 3312 and 3495). The remaining four amino acid substitutions were located in regions of the N (n = 1) or G (n = 3) proteins with no described function.

Based on the Blosum80 matrix score, 47.9% (11/23) of the amino acid changes were classified as favorable, 21.7% (5/23) were classified as neutral and 30.4% (7/23) of the amino acid

																			Second ORF P			
Viral strain	398 (N)	488 (P)	549 (P)	580 (P)	592 (P)	595 (P)	604 (P)	1068 (G)	1361 (G)	1426 (G)	1445 (L)	1526 (L)	1934 (L)	2506 (L)	2513 (L)	2941 (L)	3312 (L)	3362 (L)	3495 (L)	12 (C')	53 (C')	3 (C)	44 (C)
NJ0806VCB	**R**	**T**	**K**	**D**	**S**	**N**	**I**	**V**	**Y**	**P**	**F**	**Q**	**Q**	**S**	**Y**	**H**	**D**	**I**	**G**	**M**	**L**	**M**	**L**
NJ0612NME6	**K**	**N**	**R**	**G**	**P**	**S**	**M**	**I**	**F**	**Q**	**L**	**R**	**K**	**N**	**C**	**R**	**N**	**L**	**D**	**T**	**M**	**T**	**M**
NJ1008JAB	R	N	K	G	P	S	M	I	F	Q	L	Q	K	S	Y	H	N	I	D	M	M	M	M
NJ207512NM	K	N	R	G	P	S	M	I	F	Q	L	R	K	N	C	R	N	L	D	M	M	M	M
Colorado/21793/2014	K	N	R	G	P	S	M	I	F	Q	L	Q	K	N	C	R	N	L	D	M	M	M	M
Texas/NJ18826/20014	K	N	R	G	P	S	M	I	F	Q	L	Q	K	N	C	R	N	L	D	M	M	M	M
Texas/16313/2014	K	N	R	G	P	S	M	I	F	Q	L	Q	K	N	C	R	N	L	D	M	M	M	M
Arizona/5481555/2015	K	N	R	G	P	S	M	I	F	Q	L	Q	K	N	C	R	N	L	D	M	M	M	M
BLOSUM80 Matrix Score	2	0	2	**-2**	**-1**	0	1	3	3	**-2**	0	1	1	0	**-3**	0	1	1	**-2**	**-1**	2	**-1**	2

FIGURE 3 | Amino acid differences between viral strain NJ0806VCB (1.2) and different viral strains from lineage 1.1. Numbers in the headings of each column represent the specific locations in the concatenated coding sequence of VSNJV, and letters in parenthesis denote the corresponding viral protein where the predicted substitution occurs. The BLOSUM80 matrix score at different positions reflects the biological meaningfulness of the amino acid substitution (see "Materials and Methods" section).

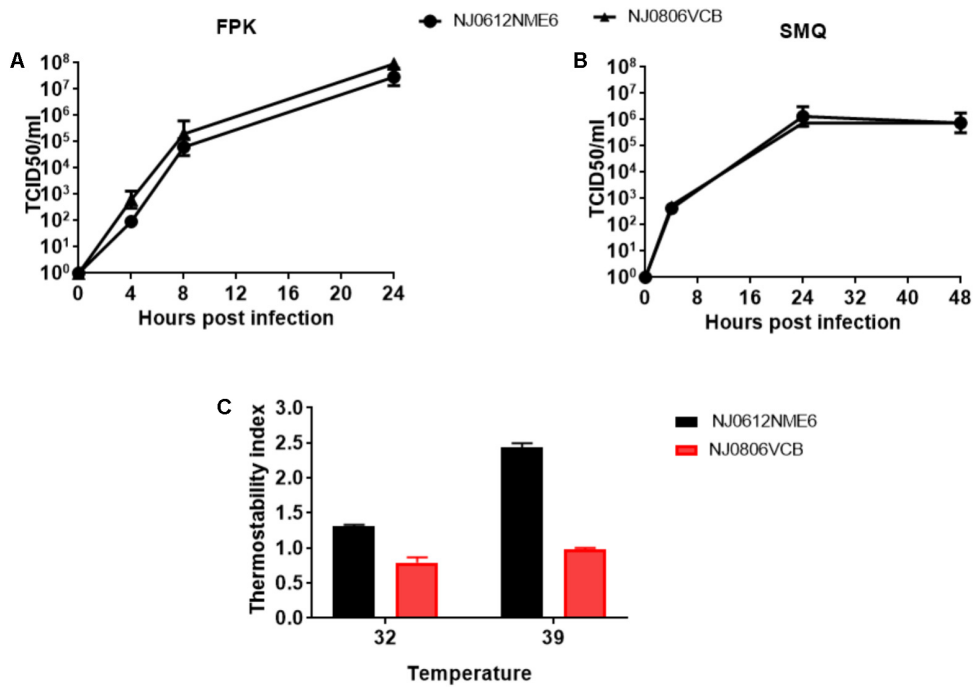

FIGURE 4 | *In vitro* growth characteristics of NJ0612NME6 and NJ0806VCB. **(A)** Fetal porcine kidney cells and **(B)** porcine macrophage cells were infected at an MOI = 0.01 and virus yields obtained at the indicated times post-infection were titrated in Vero cells. Data represents the means and standard deviations from three independent experiments. **(C)** The ability of NJ0612NME6 and NJ0806VCB to grow at 32°C and 39°C. Thermostability index was determined by the viral titer of each strain at the specific temperature divided by the titer at 37°C. Values >1 reflect higher thermostability, <1 lower thermostability and =1 no changes in thermostability.

replacements (amino acid residues in the VSNJV genome at positions 580, 592, 1426, 2513, and 3495 and C′/C positions 12 and 3) resulted in predicted changes in size, charge, or hydrophobicity (**Figure 3** and **Supplementary Figure S3**).

In vitro Growth Characterization

To explore possible biological differences between lineages 1.1 (NJ0612NME6) and 1.2 (NJ0806VCB) *in vitro* growth characteristics were evaluated in primary fetal swine cell cultures and primary swine macrophage cultures. Overall, both viruses displayed similar growth kinetics in both cell types (**Figures 4A,B**).

The ability of each strain to grow at two different temperatures (32°C and 39°C) was determined in Vero cells using a ratio of their titer at the given temperature divided by their titer at 37°C (Thermostability index). Overall, the results indicate that virus NJ0612NME6 was slightly more thermostable than virus NJ0806VCB (**Figure 4C**). The thermostability index of NJ0612NME6 (2.4 ± 0.08) was higher than that of NJ0806VCB (0.97 ± 0.031). The difference in thermostability indexes at 32°C

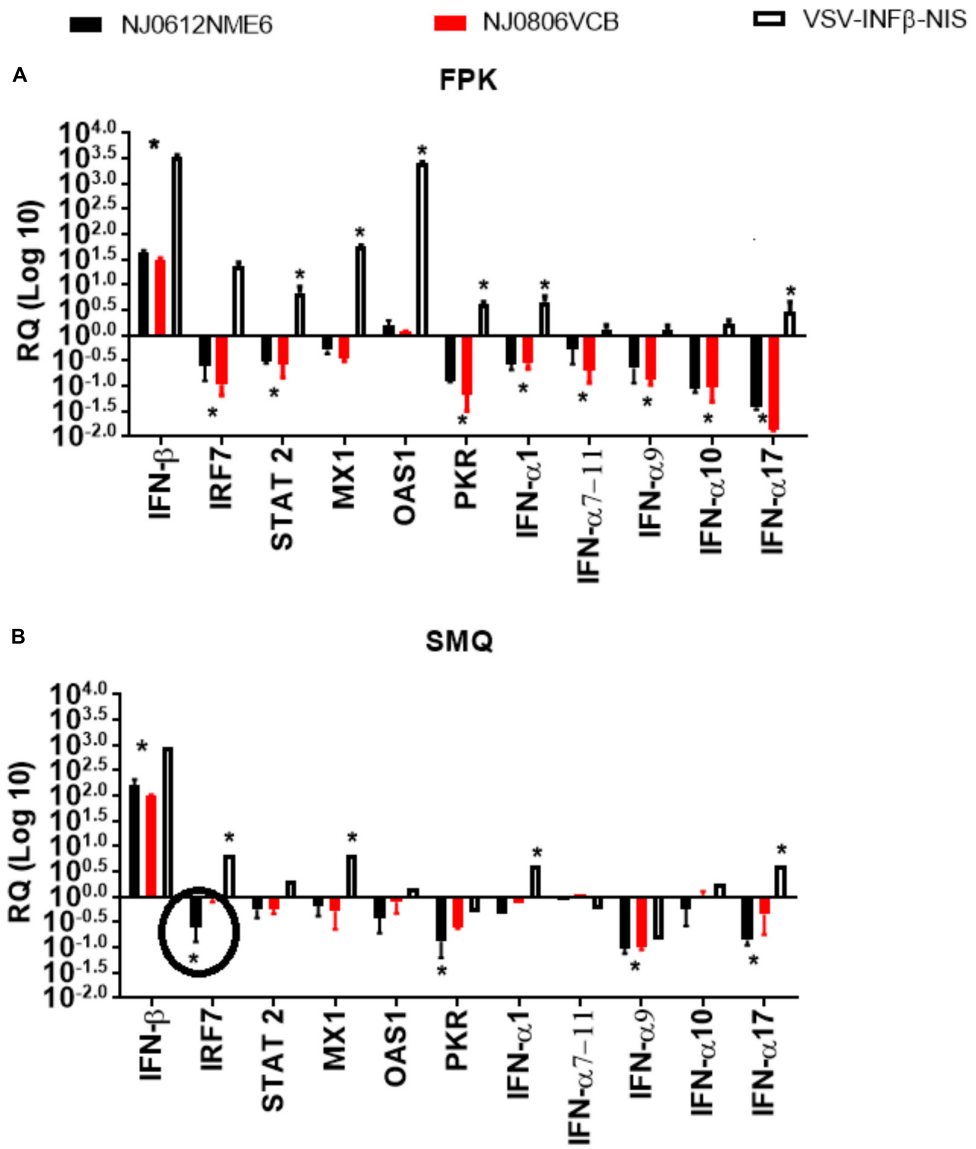

FIGURE 5 | Transcriptional regulation of the immune response. Changes in gene expression measured by rRT-PCR were determined in **(A)** fetal porcine kidney cells (FPK) and **(B)** porcine macrophage cells (SMQ). VSV-INFβ-NIS was used as a positive control. Values are represented as relative quantities (RQs) of mRNA accumulation (estimated by $2^{-\Delta\Delta C_T}$) with their corresponding SD. RQ values were considered significant when they departed from the corresponding level in uninfected cells by at least threefold in either direction. Asterisks represent significant values in one of the three viruses, and circles were used to represent significant differences between NJ0612NME6 and NJ0806VCB.

was smaller, but NJ0612NME6 had a higher thermostability index (1.3 ± 0.02) than NJ0806VCB (0.79 ± 0.10) (**Figure 4C**).

Transcriptional Regulation of the Immune Response

To evaluate the ability of each virus to regulate the antiviral immune response during *in vitro* infection, mRNA was extracted from infected cultures and the levels of 11 mRNAs were quantified by qRT-PCR to determine the ability of each strain to disrupt the transcriptional wave of innate immune gene responses during VSNJV infection (Stojdl et al., 2003). The change in normalized mRNA expression levels of a cellular gene in VSNJV infected cells was considered significant when it deviated at least threefold from its level in uninfected cells. A recombinant virus, VSIV-IFN-β-NIS, that constitutively expresses human interferon beta was used as a positive control for gene expression. In general, all three viruses stimulated the primary transcriptional wave of genes in both cell types as evidenced by the increased level of IFN-β mRNA upon infection (**Figures 5A,B**). As expected, VSIV-IFN-β-NIS induced a higher sustained level of IFN-β mRNA accumulation than the other viruses with no significant difference observed between NJ0612NME6 and NJ0806VCB. However, unlike VSIV-IFN-β-NIS, both NJ0612NME6 and NJ0806VCB disrupted the

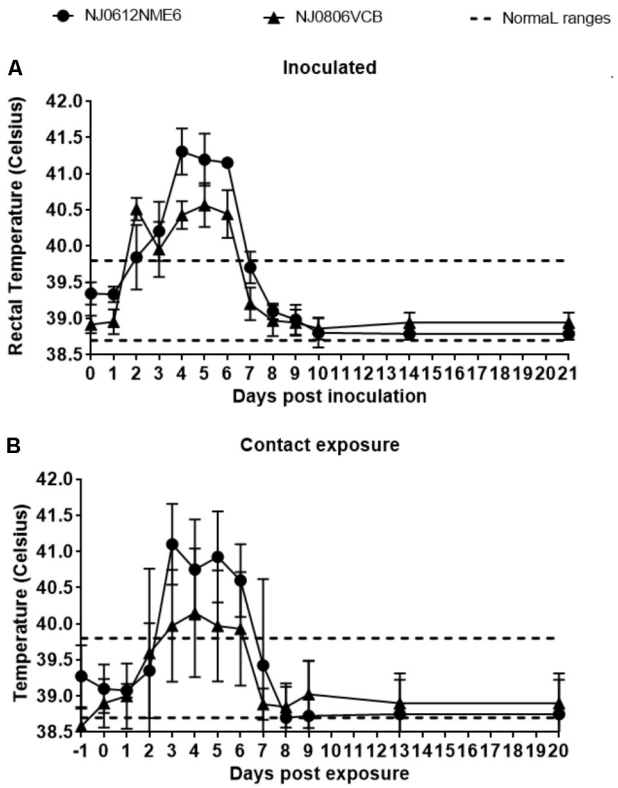

FIGURE 6 | Fever response following NJ0612NME6 or NJ0806VCB inoculation. Two independent groups of four pigs were inoculated with 1×10^7 $TCID_{50}$ and then allowed to co-mingle with four contact pigs at 24 hpi. The rectal temperature of **(A)** inoculated and **(B)** contact exposure pigs is shown along with a dashed line which represents the standard rectal temperature of an uninfected pig. In all cases values represent means and standard deviations for each group of pigs.

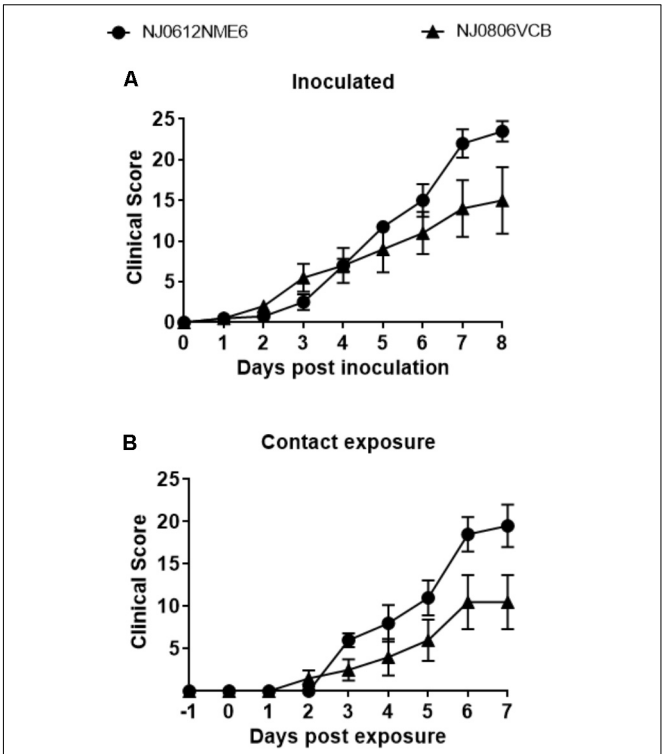

FIGURE 7 | Clinical scores following NJ0612NME6 or NJ0806VCB inoculation. Two independent groups of four pigs were inoculated with 1×10^7 $TCID_{50}$ and then allowed to co-mingle with four contact pigs at 24 hpi. Pigs were examined daily for the presence of lesions in the snout, mouth, lips, feet, and the cumulative clinical scores are given for the **(A)** inoculated and **(B)** contact exposure pigs. In all cases values represent means and standard deviations for each group of pigs.

secondary and tertiary transcriptional waves of gene expression (i.e., responses independent of IFN-β protein production). This effect was more pronounced in primary fetal swine cell cultures where a large number of down-regulated cytokine genes were detected. Although both NJ0612NME6 and NJ0806VCB were able to disrupt the innate immune cytokine wave response, NJ0612NME6 was more efficient (3.17-fold) than NJ0806VCB in down regulating the transcription of IRF7 in primary swine macrophage cultures.

Assessment of Lineage 1.1 and 1.2 Virulence in Swine

Following the experimental design described above, we utilized a previously established swine model (Martinez et al., 2003) to contrast the virulence of lineage 1.1 and 1.2 viruses. Increased body temperature (>39.8°C) was observed beginning at 2 days post infection (dpi) in directly inoculated or 3 days post exposure (dpe) in contact exposed animals and lasted until 6 dpi and 6 dpe for directly inoculated and contact exposed animals, respectively. Higher overall temperatures were observed in animals infected with NJ0612NME6 (lineage 1.1) than in those infected with NJ0806VCB (lineage 1.2) regardless of the route of infection (**Figures 6A,B**). Vesicles developed within the area of scarification in all directly inoculated pigs starting at 2 dpi for both viruses, subsequently increased in size and ruptured by 3–5 dpi. Clinical scores, based on the number of vesicular lesions in specified areas (digits, oral mucosa, lips, snout and the skin covering the carpal or tarsal joints) increased steadily from 3 to 4 dpi and peaked at 8 dpi for both viruses. Overall, average clinical scores were higher for directly inoculated (23.5 ± 2.5) and contact-exposed pigs (19.5 ± 2.5) infected with NJ0612NME6 than those observed for directly inoculated (15 ± 4.08) and contact-exposed pigs (10.5 ± 3.2) infected with NJ0806VCB (**Figures 7A,B**). Additionally, between 4 and 5 dpi an inflammatory response was observed in carpal and tarsal joints of all pigs infected with NJ0806VCB.

Viral Shedding

Viral dissemination and shedding was evaluated by rRT-PCR and virus isolation from nasal and OP swabs. No significant differences in genome copy number were found between groups of pigs infected with either virus. VSNJV RNA was detected in nasal and OP swabs as early as 1 dpi, peaked between 3 and 4 dpi, and remained at detectable levels until the end of the

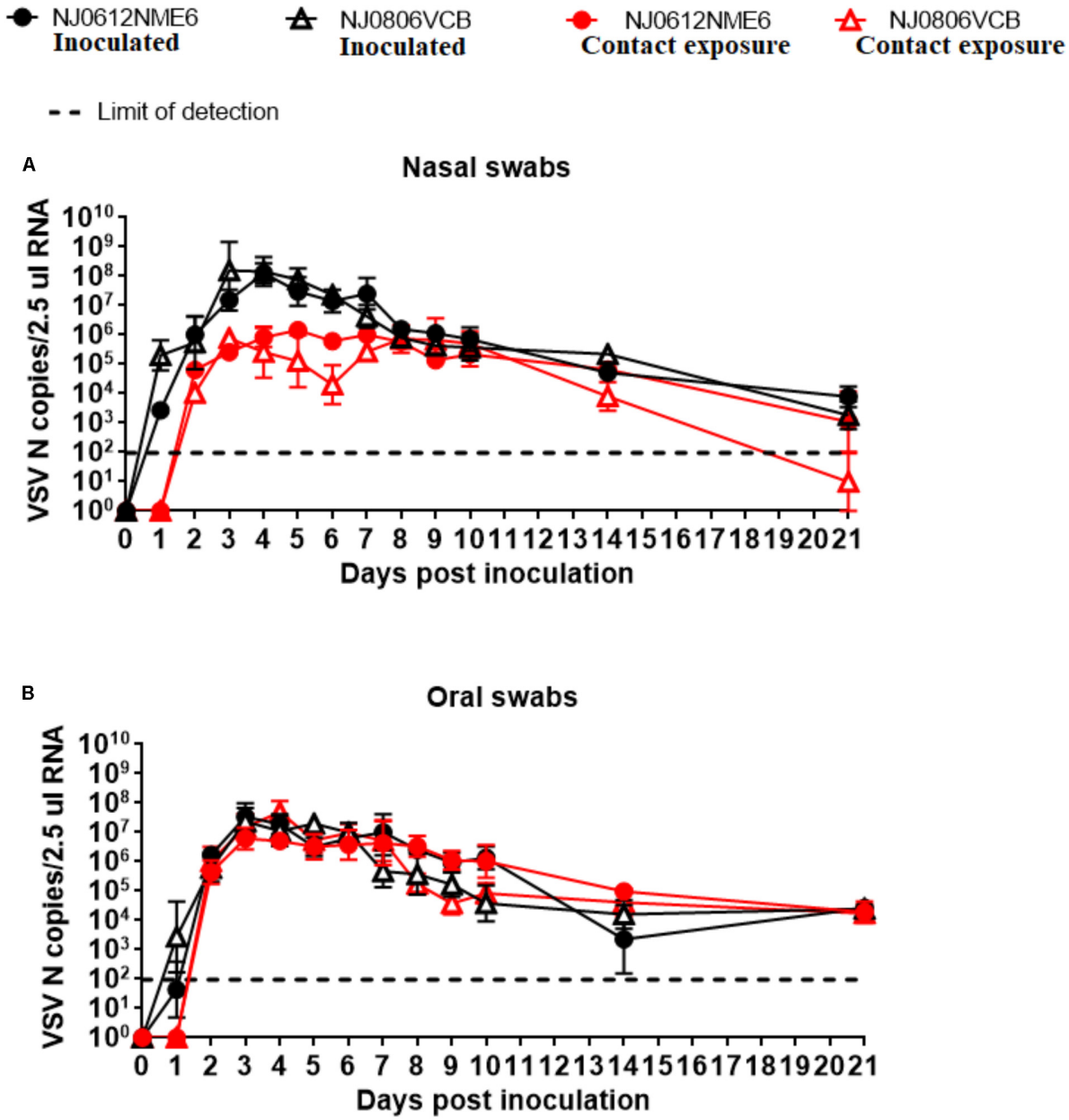

FIGURE 8 | Detection of viral RNA in clinical samples. Biological samples were collected at indicated time points after inoculation with NJ0612NME6 or NJ0806VCB and RNA was analyzed by rRT-PCR to detect VSV nucleocapsid (N) RNA in **(A)** nasal swabs or **(B)** oral swabs of both directly inoculated and contact exposure pigs.

experiment in all pigs regardless of the virus or route of infection (**Figures 8A,B**). However, an average higher RNA copy numbers (∼100-fold) were observed in nasal swabs collected between 3 and 6 dpi from directly inoculated animals versus those infected by contact exposure, regardless of the virus used (**Figure 8A**).

Infectious virus was intermittently recovered from nasal and OP swabs of pigs infected with both viruses between 1 and 6 dpi. The detection of infectious virus from nasal and OP swabs was earlier and slightly higher in pigs infected by NJ0806VCB than in the ones infected by NJ0612NME6. Overall, higher titers of infectious virus were recovered from nasal swabs than from OP swabs in both groups regardless of the virus used for the infection (**Figures 9A,B**). For nasal swabs, the peak viral titers occurred between 3 and 4 dpi and reached maximum viral titers of approximately 5.5 $TCID_{50}$/ml. This peak was consistent with the time when vesicles on the snout ruptured in directly inoculated animals, and might explain why the majority of positive viral isolations (92%), were from pigs infected by direct inoculation regardless of the virus (**Figure 9A**). Conversely, recovery of infectious virus from OP swabs was similar in all groups regardless of the route of exposure or the virus (**Figure 9B**).

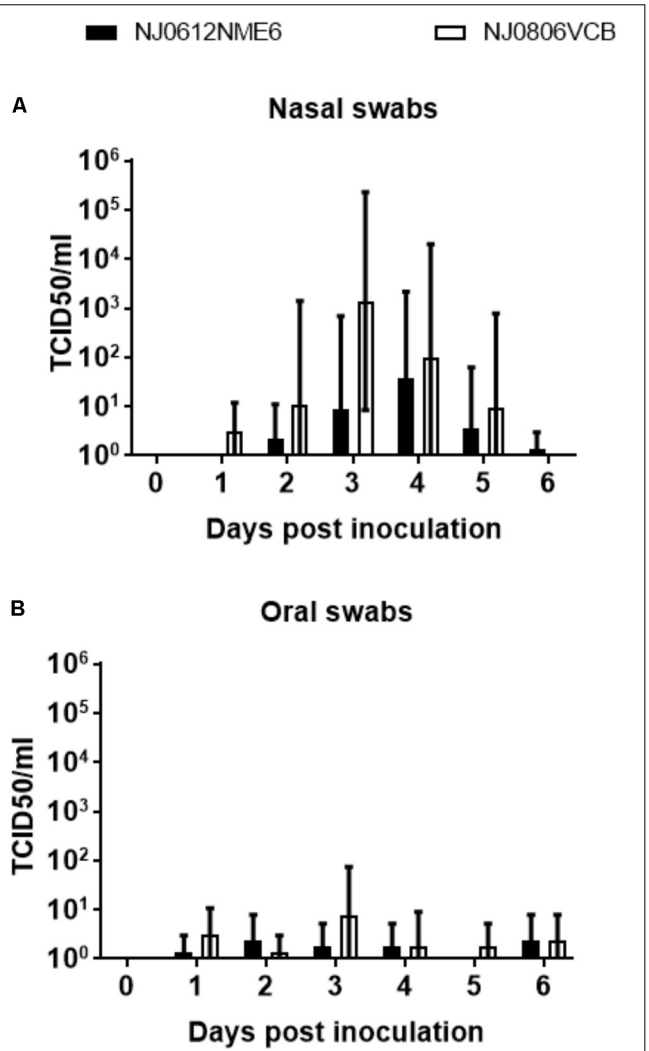

FIGURE 9 | Detection of infectious virus in clinical samples. Infectious virus was isolated in Vero cells from **(A)** nasal swabs or **(B)** oral swabs after infection with NJ0612NME6 or NJ0806VCB at expressed days post-infection. Positive isolations were confirmed by rRT-PCR and viral titrations were conducted in Vero cells. Viral titers are expressed in $TCID_{50}$/ml.

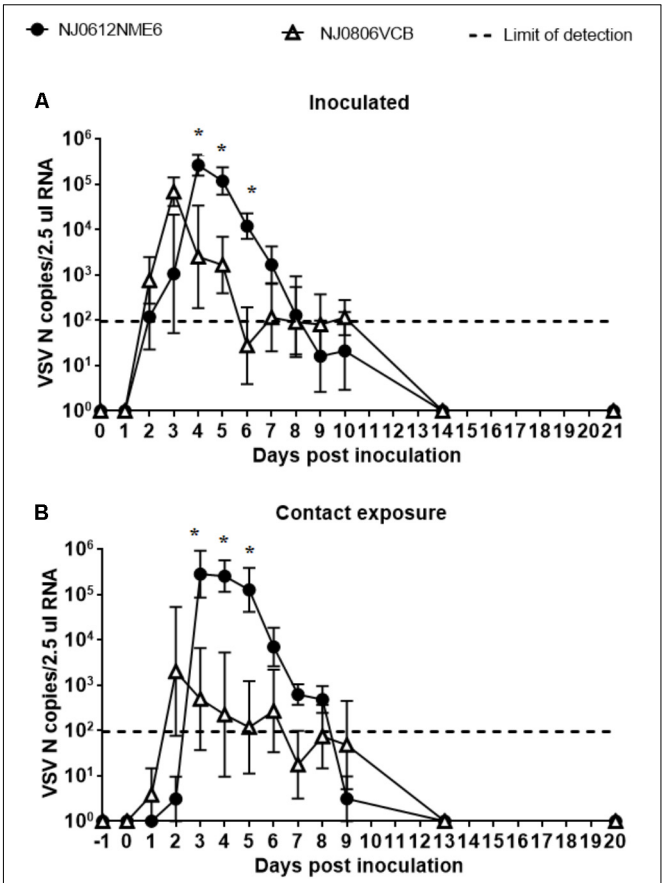

FIGURE 10 | RNAemia. VSNJV RNA in blood was detected in **(A)** inoculated and **(B)** contact exposure pigs. Dashed lines represent the limit of detection of rRT-PCR (1.98 VSV N copies/2.5 μl RNA). Values represent means and standard deviations for each group, and significantly different values ($p < 0.05$) between groups are indicated with asterisks (*).

RNAemia

To assess the presence of VSV in the bloodstream, blood samples were collected at multiple times post infection. Total RNA was isolated and evaluated by rRT-PCR to determine the number of viral genome copies. Viral RNA in blood (RNAemia) was first detectable at 2 dpi and peaked at 3 or 4 dpi depending on the viral strain (**Figures 9A,B**). In pigs infected with NJ0806VCB, RNAemia peaked earlier than in pigs infected with NJ0612NME6, regardless of exposure route. However, between 4 and 6 dpi, pigs infected with NJ0612NME6 (regardless of the route) had a significantly higher level of RNAemia ($p < 0.05$) relative to pigs infected with NJ0806VCB. Levels of RNAemia dropped gradually and fell below the limit of detection by 9 dpi (**Figures 10A,B**). Similar results were obtained using serum samples (not shown).

Despite the high levels of RNAemia in the blood and serum of infected pigs, we were unable to recover infectious virus. Multiple attempts of overlaying blood and serum samples on susceptible BHK-21 or Vero cells with varying dilutions of the blood or serum, in order to dilute out potential inhibiting factors, failed to yield infectious virus.

Neutralization of VSV by Non-immune Pig Serum

Previous reports (Beebe and Cooper, 1981; Tesfay et al., 2013, 2014) suggest that non-immune serum from humans, mice and dogs neutralize VSV by the concerted actions of IgM and complement. To explore if this was also the case for pigs, we evaluated the neutralizing activity of non-immune pig serum (NPS) against VSNJV. First, the amount of complement present in NPS was determined using a standard hemolytic assay. For this determination, the NPS was used either fresh or heat inactivated (NPSH), a condition known to inactivate complement. We found 64 complement hemolytic (CH50) units/100 μl in NPS, while minimal complement activity (0.28 CH50 units/100 μl) was

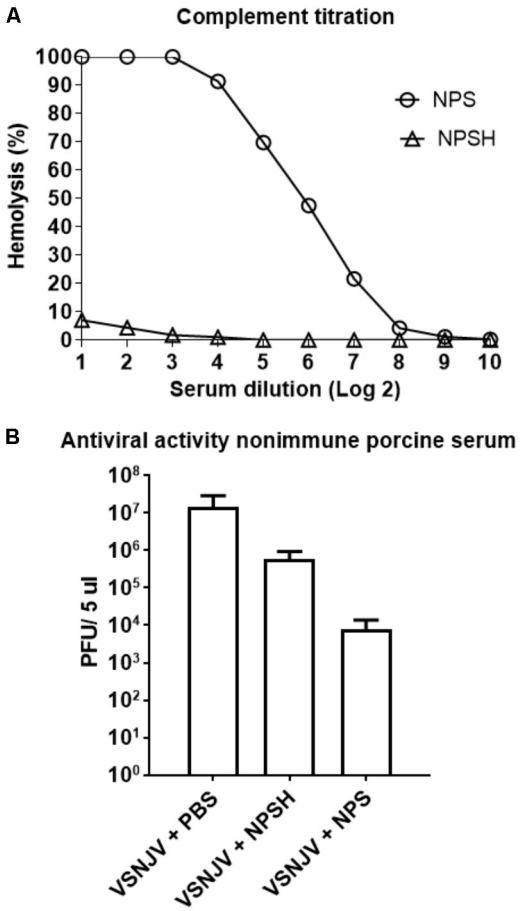

FIGURE 11 | Non-immune porcine serum (NPS) neutralizing activity. **(A)** The number of complement units in NPS and heat-inactivated NPS (NPSH) were determined by standard hemolysis technique. **(B)** The ability of NPS or NPSH to neutralize VSNJV infection was determined by plaque assay. As a negative control de virus was incubated with PBS.

detectable in heat-inactivated NPS (NPSH) (**Figure 11A**). Once we determined the presence of complement in NPS, we tested the ability of both NPS and NPSH to neutralize NJ0612NME6. Results showed that the titer of NJ0612NME6 was reduced from 7.16 ± 0.1 to 3.89 ± 0.1 $\log_{10}$ PFU (1000-fold) after 1 h of incubation with NPS compared with the untreated control (VSNJV + PBS) (**Figure 11B**). Interestingly, despite the heat inactivation, NPSH was still able to reduce the infectious titer by 10-fold (from 7.16 ± 0.1 to 5.76 $\log_{10}$ ± 0.13 PFU) (**Figure 11B**). Therefore, the non-specific viral neutralizing activity mediated by the presence of a mix of thermostable and thermolabile factors (such as complement proteins) in pig blood explains in part the failure to isolate infectious VSNJV from infected pig blood and serum.

Innate Immune Response

To assess the differences in the ability of NJ0612NME6 and NJ0806VCB to modulate the innate immune response *in vivo*, the antiviral activity and total levels of TNF and IL-6 in serum were analyzed in samples collected between 0 and 5 dpi. Serum type I interferon levels determined by Mx-CAT reporter assay showed significant ($p < 0.05$) higher levels of antiviral activity (∼82.03 and 171.81 IFN units/ml) in pigs infected with endemic virus NJ0806VCB between 2 and 3 dpi relative to pigs infected with epidemic virus NJ0612NME6 (**Figure 12A**). On the other hand, significant ($p < 0.05$) higher levels of total TNF (105.19 ± 45.40 pg/ml) were detected in pigs infected with NJ0806VCB between 4 and 5 dpi relative to pigs infected with NJ0612NME6 (**Figure 12B**). Conversely, IL-6 detectable levels (27.5 ± 8.81 pg/ml) in serum were only found in pigs infected with NJ0612NME6 between 4 and 5 dpi (**Figure 12C**) Overall, these results suggest that NJ0612NME6 has increased ability than NJ0806VCB to down regulate the innate immune response.

Adaptive Immune Response

Neutralizing antibodies against VSNJV were first detected at 5 dpi in directly inoculated and 6 dpe in contact exposed animals. Antibody titers were similar in both groups at 14 dpi. However, at 21 dpi, neutralizing antibody levels were 10-fold higher in pigs infected with NJ0612NME6. Interestingly, while the decrease of RNAemia in pigs infected with NJ0806VCB occurred before the increase in the antibody titers, the decrease of RNAemia in pigs infected with NJO612NME6 was clearly associated with the development of the antibody response (**Figures 13A,B**). In addition, we assessed the early antibody response (IgM/IgG) in sera from animals inoculated with either virus using complement fixation test (**Figure 13C**). Interestingly, negative results were obtained in both groups of animals between 0 and 4 dpi. However, between 5 and 6 dpi a 10- and 100-fold higher titers of complement fixation antibodies was observed in pigs infected with NJ0806VCB than in those infected with NJ0612NME6.

Post-mortem Examination

To determine virus distribution in tissues from pigs infected with NJ0612NME6 or NJ0806VCB, 12 selected tissues were harvested from two directly inoculated and two contact-exposed pigs from each virus group at 21 dpi and 20 dpe, respectively. Viral RNA was detected in lymphoid and epithelial tissues from lesion sites for all pigs. The popliteal lymph node (Pop-LN) was the tissue with the highest levels of viral RNA in both groups (**Figure 14**). Overall, significant ($p < 0.05$) mean detection levels of viral RNA in lymphoid tissues (PTON, SMLN, and GHLN) was greater in pigs infected with NJ0612NME6 than in those infected with NJ0806VCB. Conversely, significantly ($p < 0.05$) higher levels of viral RNA in epithelial samples (CB-V and ATONG) were detected in pigs infected with NJ0806VCB. Interestingly, even with the high amount of VSNJV RNA found in most of these tissues, infectious virus was not recovered from any of the tissue samples collected post-mortem.

DISCUSSION

In this study, we determined distinctive molecular and biological characteristics associated with the increased virulence in pigs of an epidemic, emergent VSV viral strain from lineage 1.1 and

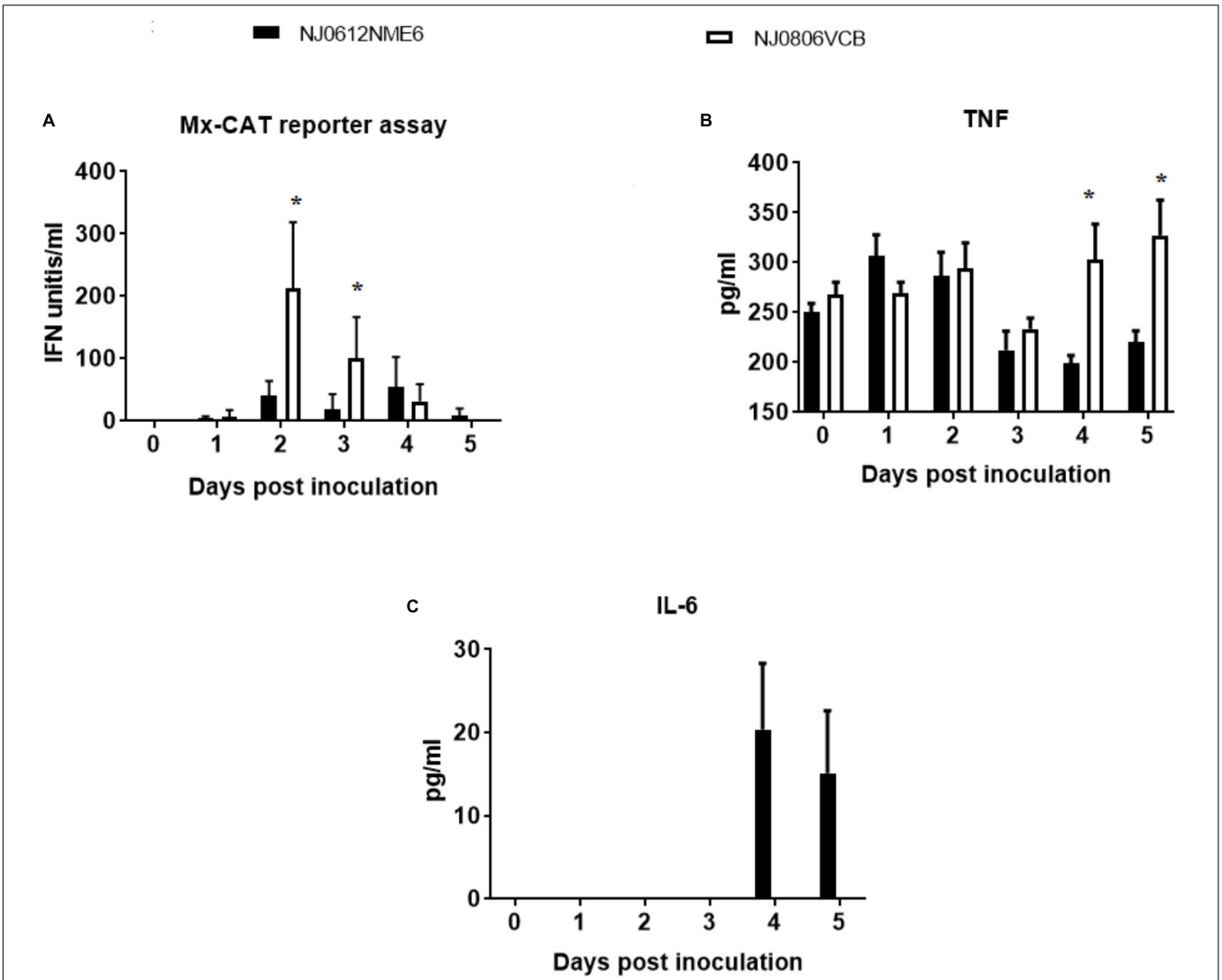

FIGURE 12 | Innate immune response following infection in pigs with NJ0612NME6 or NJ0806VCB. Serum samples collected from inoculated during the acute phase of the infection was used to evaluate: **(A)** Type I IFN antiviral activity by MX-CAT reporter assay, **(B)** tumor necrosis factor (TNF), and **(C)** interleukin 6 (IL-6) by ELISA. Significantly different values ($p < 0.05$) between groups are indicated with asterisks (*).

contrasted these results to those obtained with a closely related endemic viral strain from lineage 1.2 (Velazquez-Salinas et al., 2014). These strains were compared using a combination of bioinformatics tools, various *in vitro* assays as well as *in vivo* pathogenesis experiments in pigs, a natural vertebrate host of this virus. Results indicated that these strains differ in their ability to cause disease in pigs, and that these differences might be related to the increased capability of epidemic strains to modulate the host innate immune response.

Our comparative analysis identified multiple genomic differences that might be related to the increased virulence of epidemic strain NJ0612NME6. A previous report showed that multiple substitutions in the Venezuelan equine encephalitis virus genome were responsible for the increased virulence associated with the emergence of epizootic strains (Greene et al., 2005).

The high number of dS substitutions observed within lineage 1.1 suggests that during the evolution of this epidemic strain, dS sites in the genome are evolving under positive selection. This pattern of evolution is consistent with populations of VSV adapting to specific conditions (Novella et al., 2004). Similar to our analysis, a previous study of natural populations of arboviruses supported the relevance of dS substitutions related to the specific codon usage in their respective hosts (Velazquez-Salinas et al., 2016). Furthermore, a previous study showed that the experimental introduction of dS substitutions in the L gene of VSV decreased virulence in mice (Wang et al., 2015). Comparing the differences in virulence between the two viral lineages tested in our study, a possible role of dS substitutions on viral virulence should be considered.

Very little is known about the role of specific amino acid replacements in VSV virulence in natural hosts. One of the few

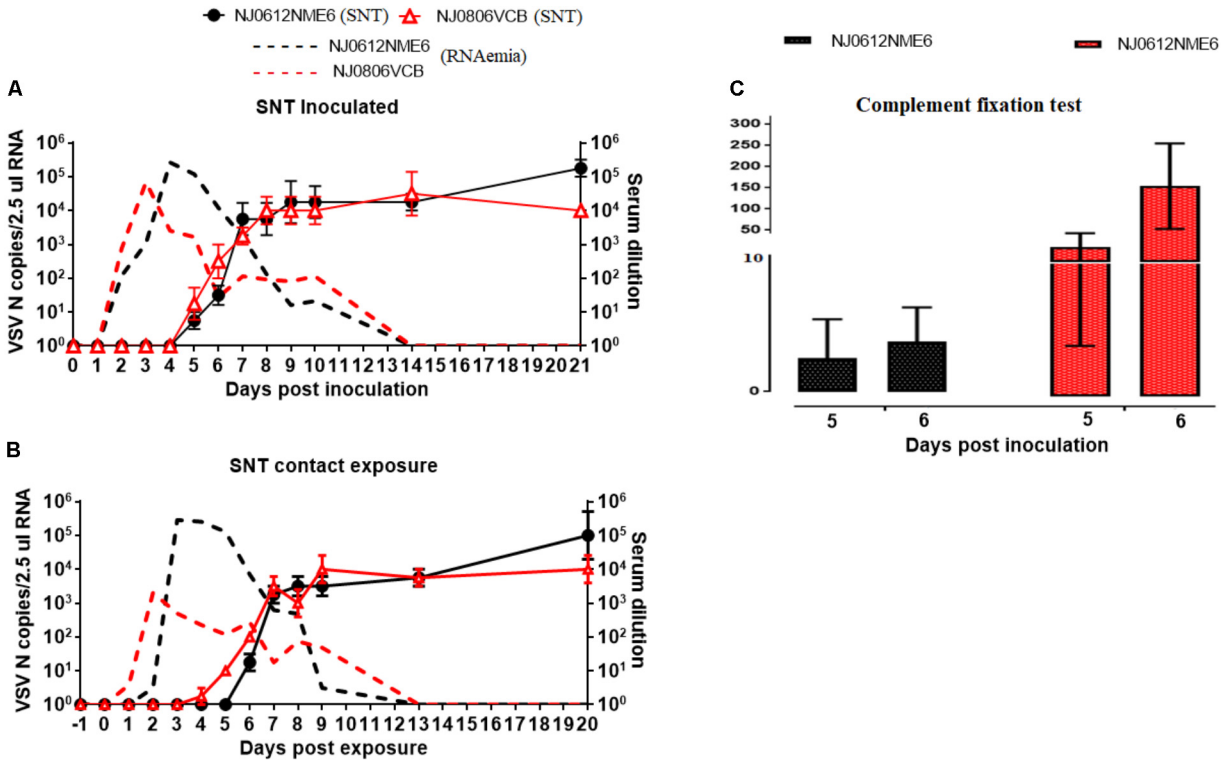

FIGURE 13 | Adaptive immune response. Serum neutralization antibody titers following infection with NJ0612NME6 or NJ0806VCB. Adaptive immune response was monitored by measuring anti-VSV antibodies in serum collected at indicated the time points in **(A)** inoculated or **(B)** contact pigs. The broken lines in black and red represent levels of viral RNA in the blood of pigs infected with NJ0612NME6 and NJ0806VCB, respectively, indicating a negative correlation between antibody immune response and RNAemia phase. **(C)** The early antibody response in infected pigs with different viruses was evaluated by complement fixation test.

proteins known to influence VSV virulence is the M protein, which has been recognized as a major immune regulatory protein for its ability to suppress IFN-bβ gene expression (Ferran and Lucas-Lenard, 1997; Ahmed et al., 2003). However, our results showed identical deduced amino acid sequences for the M proteins between the lineages 1.1 and 1.2 representative viruses, indicating that other genomic determinants might be mediating virulence.

Of the 19 predicted amino acid substitutions located in the viral structural proteins of the epidemic strain, 18 were located in the proteins P, G, and L. Interestingly, despite the P protein constituting only ∼7.7% of the total coding sequence, almost half of the total predicted amino acid substitutions between the two viruses were located within this protein. Very little is known about the role of the P protein in VSV virulence. A recent study conducted with spring viremia of carp virus (another vesiculovirus) showed the ability of the P protein to regulate the immune cellular response by decreasing IFN production (Li et al., 2016). This is consistent with the evolutionary role of this protein to antagonize IFN activities in other Rhabdoviruses such as rabies virus (Rieder and Conzelmann, 2011; Okada et al., 2016) and makes the P protein an interesting target for future studies of VSV virulence.

The VSV G protein has been described as a determinant of virulence in swine (Martinez et al., 2003). Previous studies have shown the ability of the G protein to trigger type I IFN secretion *in vitro* (Georgel et al., 2007; Janelle et al., 2011). Interestingly, amino acid substitutions at residues 1068 (V→I) and 1361 (Y→F) reported in our study are in a region of G that influences IFN secretion (Georgel et al., 2007; Janelle et al., 2011). This might explain the decreased serum levels of systemic antiviral activity and the increased virulence in pigs infected with the epidemic strain.

Here, we report the ability of lineage 1.1 (NJ0612NME6) to produce a higher number of vesicular lesions in pigs relative to NJ0806VCB, its closest endemic relative. Our results support previous hypotheses that differences in severity of clinical signs may have a direct impact on viral spread during epidemics by increasing animal-to-animal transmission as well as virus availability for insect-vectored transmission (Smith et al., 2012). This is also consistent with previous studies highlighting the importance of vesicular lesions as a primary source of infectious virus during transmission (Stallknecht et al., 2001). Another differential clinical finding was the ability of NJ0612NME6 to induce a higher febrile process relative to NJ0806VCB. Interestingly, *in vitro* replication of VSV at temperatures above 40°C represents a selective factor favoring the emergence of variants with an increased capacity to evade the antibody neutralization activity by polyclonal sera of VSV infected animals, suggesting the ability of VSV to avoid the immune repose during

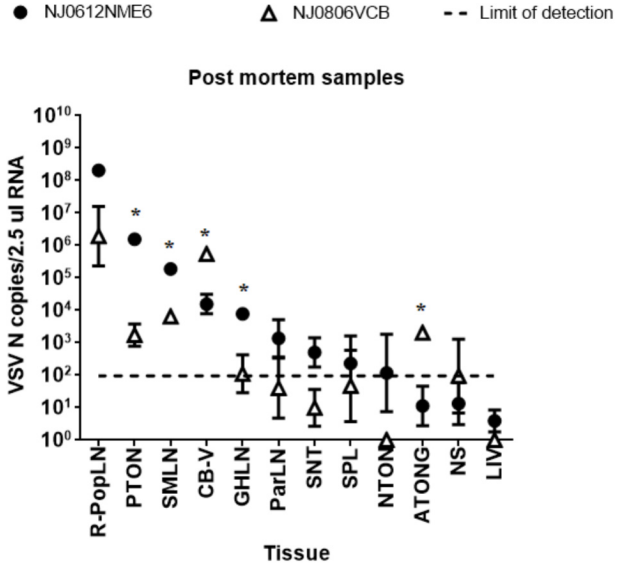

FIGURE 14 | Detection of viral RNA in postmortem samples. RNA from tissues collected at 21 dpi was analyzed by rRT-PCR to detect VSV nucleocapsid RNA. The dashed line represents the limit of detection of rRT-PCR (1.98 VSV n copies/2.5 μl RNA). Values represent means and standard deviations for four pigs (two inoculated and two contact) from each group. Significant differences ($p < 0.05$) between groups are indicated by asterisks (*).

febrile periods (Presloid et al., 2016). This is an interesting concept considering our *in vitro* results that suggested that lineage 1.1 has a slightly increased ability to grow at higher temperatures than lineage 1.2. This ability could be linked to amino acid differences between both strains in the G protein. Moreover, it might help to explain the increased clinical scores observed in pigs infected with virus NJ0612NME6 despite their higher febrile response compared to pigs infected with the endemic virus. This observation might encourage further studies aimed at understanding the role of specific residues in the G protein relative to virus thermostability and how increased thermostability might impact viral spread during epidemics.

Importantly, this is the first report demonstrating the presence of viral RNA in serum and blood samples of pigs experimentally infected with VSV. This finding is consistent with previous reports describing virus RNA in cattle and horses naturally infected with VSV (Tolardo et al., 2016), and horses experimentally infected with VSIV (Howerth et al., 2006). In fact, VSV RNA was regularly detected in serum samples from cattle naturally infected in Mexico with a viral strain associated with lineage 1.1 (Velazquez-Salinas, unpublished data).

Although previous reports indicate the absence of viremia during experimental VSV infection of pigs (Comer et al., 1995a,b; Stallknecht et al., 1999), our results suggest the existence of a viremic phase during the acute infection of pigs that might be masked by the inhibitory effect of heat stable and thermolabile serum proteins. Despite our inability to isolate infectious virus from blood and serum, our results indicated that the higher levels of viral RNA found in the blood of pigs infected with NJ0612NME6 correlated well with higher fever and clinical scores relative to pigs infected with NJ0806VCB, and suggest that differences in blood RNA levels during the acute phase of the infection might be used as a virulence marker in future pathogenesis studies in pigs.

We showed that infection of pigs with an epidemic or endemic VSV induced disparate serum levels of IgM/IgG antibodies, systemic antiviral activity, TNF and IL-6 during the acute phase, as well as marked differences in virulence. The earlier development of antibody response (determined by CFA) in pigs infected with virus NJ0806VCB compared to those infected with virus NJ0612NME6 constitutes an important finding in our study and might help to understand differences in virulence. The role of IgG/IgM antibodies in serum of different species have been shown to play an important role in neutralizing VSV by activating the classical complement pathway (Beebe and Cooper, 1981; Tesfay et al., 2013, 2014). One possible explanation for the faster IgM/IgG response seen in pigs infected with NJ0806VCB might be associated with the higher type I IFN response observed in these pigs, since type I IFN has been previously associated with the increase of primary antibody responses during VSV infections in a mice model (Fink et al., 2006; Fuertes et al., 2013).

The decreased levels of antiviral activity, attributed to type I IFN, in pigs infected with the epidemic strain provide a plausible explanation for the increased virulence observed in this strain, as type I IFN has been shown to have a key role in clearing VSV infection in mice (Muller et al., 1994). A recombinant VSV expressing human interferon beta was shown to be fully attenuated in experimental infection of healthy pigs, promoting the expression of multiple interferon stimulated genes (Velazquez-Salinas et al., 2017b). Our *in vitro* pathogenesis results showed that epidemic VSV was able to down-regulate the transcription of IRF-7, considered a master up-regulator of the interferon response (Ning et al., 2011). This provides a possible mechanism for the decreased antiviral activity detected during *in vivo* studies. Unfortunately under the conditions of our experiments and based on the intrinsic ability of VSV to interrupt the translation in the cell by blocking export of mRNA to the cytoplasm, we couldn't appreciate the biological effects produced by the down regulation of IRF-7 observed in our study.

On the other hand, the decreased levels of TNF in pigs infected with the epidemic VSV may also contribute to the increased virulence. This cytokine can induce the expression of interferon stimulated genes independently of the known mechanism described by the IFN JAK-STAT cascade (Wang et al., 2016). A previous study showed that systemic circulation of this cytokine in infected mice with VSV results in activation of the innate and adaptive immune responses, and this plays a critical role for survival (Shinde et al., 2018). Furthermore, cell cultures previously treated with TNF were able to induce antiviral activity against VSV in a dose dependent manner (Mestan et al., 1988).

Finally, the increased levels of IL-6 found in the serum of pigs infected with the epidemic strain might explain the higher body temperatures observed in these pigs. This cytokine is a potent endogenous pyrogen, and its systemic circulation in patients infected with other arboviruses like Chikungunya virus and

Crimean-Congo hemorrhagic fever virus, has been associated with the infection of highly virulent strains. In fact, IL-6 is considered a biomarker of virulence during the infection with these viruses (Ergonul et al., 2006; Ng et al., 2009). A previous study in mice showed that IL-6 plays a key role regulating the differentiation of dendritic cells *in vivo*, inhibiting the T-cell immune response (Park et al., 2004). The ability of IL-6 to negatively affect the T-cell response might be related to the increased levels of virulence observed in pigs infected with the epidemic strain. Therefore, it is possible that differential levels of IFN, TNF and IL-6 among pigs infected with the epidemic and endemic strains may explain the increased virulence observed with the epidemic strain.

CONCLUSION

Our results showed important molecular and biological differences between endemic and epidemic strains of VSV. These differences include the epidemic strain's increased ability to modulate innate immune responses during infection and its increased virulence compared to the endemic strain. The increased virulence in the vertebrate host could increase virus availability for animal-to-animal and vector transmission and might help explain the successful spread of this strain into non-endemic regions.

AUTHOR CONTRIBUTIONS

LV-S, SP, CS, JP, MB, AV-R, JA, and LR conceived and designed the experiments. LV-S, SP, and EO performed the experiments. LV-S, SP, and LR analyzed the data. LR, MB, and JA contributed the reagents, materials, and analysis tools. LV-S, SP, CS, MB, AV-R, JA, and LR wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01891/full#supplementary-material

FIGURE S1 | Equal sensitivity of rRT-PCR to detect VSV viruses 1.1 and 1.2.

FIGURE S2 | Synonymous substitution pattern on different genes associated with viral lineage 1.1.

FIGURE S3 | Amino acid chemical properties.

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CAPÍTULO 7

Validación de la técnica de clonaje de recombinación sito específica para la producción de una clona de cDNA de la cepa epidémica del VSNJV NJ0612NME6.

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The Role of Interleukin 6 During Viral Infections

Lauro Velazquez-Salinas[1,2], Antonio Verdugo-Rodriguez[2], Luis L. Rodriguez[1] and Manuel V. Borca[1]*

[1] Foreign Animal Disease Research Unit, USDA/ARS Plum Island Animal Disease Center, Greenport, NY, United States,
[2] College of Veterinary Medicine and Animal Science, National Autonomous University of Mexico, Mexico City, Mexico

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Dirk Dittmer, University of North Carolina at Chapel Hill, United States

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Byung S. Kim, University of Northwestern – St. Paul, United States

****Correspondence:***
Lauro Velazquez-Salinas
lauro.velazquez@ars.usda.gov

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Our recently published research on the characterization of vesicular stomatitis virus (VSV) pathogenesis in swine, identified a systemic upregulation of interleukin 6 (IL-6) during the acute phase of infection (Velazquez-Salinas et al., 2018). This upregulation was observed during infection with a highly virulent VSV strain, suggesting a potential association between IL-6 levels and virus virulence in pigs. In this opinion note we would like to explore in more detail the biological functions of IL-6 in different virus models, and present our perspective regarding the debatable role of IL-6 during viral infections. While several studies show the essential role of IL-6 to mount a proper immune response during some viral infections, others link this cytokine with exacerbation of viral disease. These latter findings lend support to the hypothesis that upregulation of IL-6 during certain viral infections may promote virus survival and/or exacerbation of clinical disease.

IL-6 is a pleotropic cytokine produced in response to tissue damage and infections (Tanaka et al., 2014). Multiple cell types including fibroblasts, keratinocytes, mesangial cells, vascular endothelial cells, mast cells, macrophages, dendritic cells, and T and B cells are associated with the production of this cytokine (Mauer et al., 2015). After targeting its specific receptor, IL-6 starts a cascade of signaling events mainly associated with the JAK/STAT3 activation pathway (Wang et al., 2013) promoting the transcription of multiple downstream genes associated with cellular signaling processes, including cytokines, receptors, adaptor proteins, and protein kinases (Pim-1, LDL-receptor, GADD45 beta, SOCS1, MAP3K8, SOCS3, GLUT3, HB-EGF, ICAM1, Mx1, PTP4A3, SGK, Pim-2, RHOBTB3, cAMP-GEFII, PDGF-receptor alpha, MLCK). It also controls the production of proteins implicated in regulation of gene expression (Blimp1, id-2H, MAFF, TTP, C/EBP-beta, SRY, TCF8, c-jun, junB, Bcl-3, Bcl-5, DEC1, Nmi, Stat1, eIF5, OBF-1, Oct-2, Stat3; Brocke-Heidrich et al., 2004). The number of genes regulated by IL-6 activity may explain the pleotropic nature of this interleukin. Accordingly, the biological consequences of IL-6 production have been associated with both pro- and anti-inflammatory effects (Scheller et al., 2011), highlighting IL-6's pivotal role in the activation and regulation of the immune response. Biological activities affected by production of IL-6 include: control of the differentiation of monocytes into macrophages by regulating the expression of macrophage colony-stimulating factor (Chomarat et al., 2000), increasing B-cell IgG production by regulating the expression of IL-21 (Yang et al., 2016), negative regulation of dendritic cell maturation by activation of the STAT3 signaling pathway (Park et al., 2004), as well as the promotion of the Th2 response by inhibiting Th1 polarization (Diehl and Rincon, 2002). Two different mechanisms have been described to promote the inhibition of Th1 polarization by IL-6: (1) IL-6 stimulates CD4 T cells to secrete IL-4 and direct the response to Th2, and (2) IL-6 affects the secretion of IFNγ by CD4 T cells, an essential interferon to promote Th1 polarization. A similar effect is produced in Th1 cells, where inhibition of IFNγ secretion in these cells affects CD8 T cell activation (Dienz and Rincon, 2009; Green et al., 2013).

Moreover, in combination with the transforming growing factor beta, IL-6 induces the differentiation of naïve CD4 into Th17 cells, which are important for the defense against pathogens at mucosal sites (Guglani and Khader, 2010). Also, IL-6 synergic interactions with IL-7 and IL-15 induce the differentiation and cytolytic capacity of CD8 T cells (Cox et al., 2013). Importantly, IL-6 is a potent pyrogenic cytokine, and has an essential role organizing lymphocyte trafficking to lymphoid organs during febrile events (Evans et al., 2015).

In addition to its roles modulating the host immune response, IL-6 has been implicated in the progression of several virus infectious. IL-6 is considered one of the most important cytokines during an infection, along with interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF-a; Dienz and Rincon, 2009). Direct evidence supporting the importance of IL-6 during viral infections has been gathered in experimental infections using IL-6-deficient mice. Using this model, IL-6 has been shown to be essential for survival of mice infected with influenza virus by promoting optimal regulation of the T-cell response, inflammatory resolution, tissue remodeling promoting lung repair, migration and phagocytic activities of macrophages, preventing viral-induced apoptosis in lung epithelial cells, and regulation of IgG isotype switching (Lauder et al., 2013; Yang et al., 2017). Other reports have also emphasized the importance of IL-6 during virus infections. Disruption of the IL-6 gene in mice infected with vaccinia virus impaired the immune response by reducing the activity of specific cytotoxic T-cells, while murine infection with VSV impaired the production of specific IgG antibodies (Kopf et al., 1994). Additional evidence of IL-6's function during a virus infection was observed during lymphocyte choriomeningitis virus infection of mice where IL-6 and/or IL-6R activity was blocked using specific monoclonal antibodies. In this model, T helper and B-cell responses were reduced during the late stages of infection, negatively affecting viral clearance (Harker et al., 2011).

Genetically engineered rabies virus carrying the IL-6 gene in its genomic backbone has been used as an alternative model of experimentation to assess the relevance of IL-6 during viral infections (Luo et al., 2018). Normal mice infected with this virus showed a higher resistance to the viral infection compared with mice infected with the parental virus. Animals infected with the engineered virus had an increased blood-brain barrier permeability with a higher number of specific CD8-T and B-cells, increased levels of circulating neutralizing antibodies, and an intensified innate immune response in the brain as defined by up-regulation of multiple interferon-stimulated genes (ISG15, ISG20, OAS1, OAS2, and MX2).

As a warning signal during viral infections, different immune cellular pathogen recognition receptors, including toll-like receptors (TLR:2, 3, 4, 7, 8, and 9), nucleotide-binding oligomerization domain-like receptors, DNA receptors, and retinoic acid-inducible gene-1-like receptors, are able to sense a variety of pathogen-associated molecular patterns displayed by viruses (envelope glycoproteins, single and double-stranded RNA, and unmethylated CpG DNA), which stimulate transcription of IL-6 among other proinflammatory cytokines (Kawai and Akira, 2010; Tanaka et al., 2014). In this context, it has been shown that specific amino acid substitutions in a TLR-like structure in the NS4B protein of a highly virulent classical swine fever virus (CSFV) strain resulted in a completely attenuated phenotype in pigs. Infection of pigs with this mutant CSFV was characterized by the sustained accumulation of IL-6 in tonsils. Further *in vitro* experiments using exogenous IL-6 confirmed the ability of this cytokine to repress the replication of CSFV in swine peripheral blood mononuclear cells, the natural target cell during CSFV infection in pigs (Fernandez-Sainz et al., 2010).

Similarly, evidence of the antiviral effect of IL-6 was described during *in vitro* studies conducted with hepatitis B virus (HBV) where the direct ability of exogenous IL-6 to suppress the replication of this virus was described. Disruption of HBV replication was characterized by a marked decrease in the number of viral genome-containing nucleocapsids, an effect mediated in an interferon-independent manner (Kuo et al., 2009). Furthermore, IL-6 was able to block HBV infection in hepatocytes by inhibiting expression of HBV receptor in the human liver, i.e., the bile acid transporter Na (+)/taurocholate co-transporting polypeptide (Bouezzedine et al., 2015), and effectively disrupted epigenetic control of the nuclear cccDNA mini-chromosome, inhibiting HBV transcription (Palumbo et al., 2015) and the expression of hepatocyte nuclear transcription factors 1 and 4 alpha (Hosel et al., 2009).

However, experimental scientific evidence also suggests potential negative consequences that increased levels of IL-6 might have on the cellular immune response against viruses. In this context different potential mechanisms involving this cytokine might affect viral clearance, ultimately favoring the establishment of a viral persistent state in infected hosts.

First, *in-vitro* secretion of IL-6 by activated splenocytes, as a consequence of stimulation of toll like receptor 1/2 by the agonist P3C, inhibited effector CD8 T-cell responses by impairing the production of interferon gamma (IFN-γ) when compared with similarly activated and stimulated splenocytes from IL-$6^{-}/^{-}$ mice (Wu et al., 2015). Similar results were obtained providing an exogenous source of IL-6, confirming the ability of IL-6 to negatively regulate effector CD8 T-cell response after T cell activation. This inhibition was orchestrated through the STAT3 signaling pathway producing the upregulation of suppressor of cytokine signaling (SOCS3) that reduces the STAT4 phosphorylation pathway induced by IL-12, which is essential for effector CD8-T cell differentiation (Wu et al., 2015). Furthermore, *in vivo* blockage of IL-6 using a monoclonal antibody during acute infection in mice with murine leukemia virus resulted in reduced viral loads, and increased production of IFN-γ and the serine protease granzyme B (essential to produce apoptosis in target cells; Wu et al., 2015).

Second, the synergistic interaction between IL-6 and interleukin 17 (IL-17) have been associated with viral persistence and exacerbated clinical outcome during infection with Theiler's murine encephalomyelitis virus (TMEV). Genetically engineered mice carrying a human IL-6 transgene have excessive production of IL-6, leading to increased production of Th17 cells during an immune response. The IL-6 and IL-17

synergistic interaction leads to induction of anti-apoptotic molecules (Bcl-2 and Bcl-xL) inhibiting the destruction of TMEV-infected cells by virus-specific CD8+ T-cells, therefore favoring virus survival (Hou et al., 2014). Also, inhibition of apoptosis by IL-17 seems to be associated with the ability of this cytokine to block the Fas-FasL pathway (Hou et al., 2009). Interestingly, the induction of immunopathology, prevention of Th1 cells, and the inhibition of IL-2 and IFN-γ production have been mentioned as potential detrimental factors induced by Th17 cells during viral infections caused by influenza virus, Mouse hepatitis virus, hepatitis C virus, herpes simplex virus, and coxsackie virus B3 (Martinez et al., 2012).

The last potential mechanism links IL-6 with the negative co-stimulator molecules programmed cell death one (PD-1) and its ligand (PDL-1). Under normal conditions, PD-1 and PDL-1 prevent autoimmunity by inducing T-cell regulation and maintaining self-tolerance (Bardhan et al., 2016). However, during chronic viral infections, T-cell ligation of PD-1 by PDL-1, expressed on infected cells, alters immunity against viruses by preventing T-cell generation and expansion (Bardhan et al., 2016). Experimental evidence evaluating the induction of PD-1 and PDL-1 after infection with TMEV in normal and transgenic IL-6 mice showed that the excessive production of IL-6 displayed by transgenic mice after infection positively correlates with increased up-regulation of PD-1 and PDL-1 molecules in the central nervous system, and consequently with reduced CD8+ cytolytic function (Jin et al., 2013). Interestingly, PD-1 and PDL-1 up-regulation appeared to be the result of the cooperative action between IL-6 and interferon type I, with IL-6 essential to the expression of maximum levels of PDL-1 (Jin et al., 2013).

Evidence from clinical studies in humans and animals have also linked the increased systemic levels of IL-6 with the exacerbation of clinical outcomes involving viral pathogens. In this context, increased levels of IL-6 in serum has been reported in human patients chronically affected with

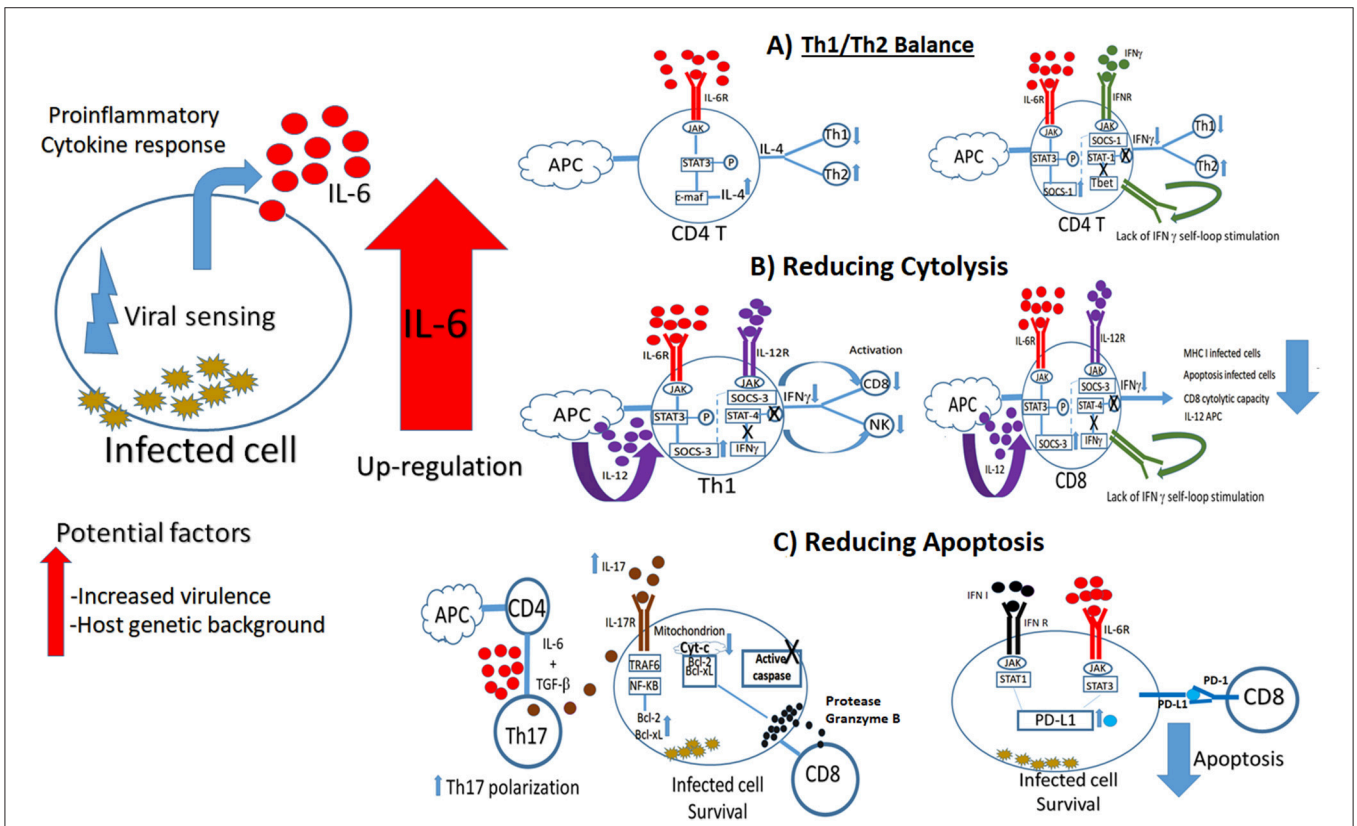

FIGURE 1 | Overexpression of IL-6 and its potential negative consequences on the viral immune response. Current scientific evidence supports different scenarios where imbalance on the IL-6 production after viral infection can affect viral clearance, promoting viral persistence and chronic infections. **(A)** IL-6 might favor Th2 polarization by stimulating STAT3 pathway, and consequently increasing the production of IL-4, and the suppressor of cytokine signaling one protein (SOCS-1). SOCS-1 affects STAT 1 phosphorylation, impairing IFNγ production by decreasing IFNγ self-loop stimulation. **(B)** IL-6 might impair cytolysis by inducing the production of SOCS-3, affecting phosphorylation of STAT 4, and consequently impairing IFNγ production, an essential IFN type II interferon molecule to promote CD8 and NK cells activation. **(C)** IL-6 might promote infected cell survival by inducing apoptosis. Overexpression of this cytokine increments Th17 polarization, increasing IL-17 production in the cellular environment. IL-17 pathway induce the production of the anti-apoptotic B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma extra-large (Bcl-xL) proteins, which prevent mitochondrion to produce the cytochrome complex protein (Cyt-c) after stimulation by the pro-apoptotic molecule protease Granzyme B, impairing the production of active apoptotic caspase molecules. An additional scenario includes the production of the programmed death ligand 1 protein (PD-L1) by the concerted action of IFN type I and IL-6. Matching between PD-L1 and programed death protein 1 (PD-1) switches off apoptosis mediated by CD8 T cells.

Andes virus (Angulo et al., 2017), influenza virus (Zheng et al., 2017), HBV (Torre et al., 1994), hepatitis C virus (Spanakis et al., 2002), human immunodeficiency virus (HIV; Borges et al., 2015), Crimean-Congo hemorrhagic fever virus (Ergonul et al., 2017), and Chikungunya virus (Chirathaworn et al., 2013). Similar results have been reported in pigs and ponies infected with VSV and influenza virus, respectively, where the virulence of different strains might be positively correlated with both local and systemic detection of IL-6 (Wattrang et al., 2003; Velazquez-Salinas et al., 2018). Additionally, transcriptome analysis of persistently infected pharyngeal tissues collected from cattle with foot and mouth disease virus showed a local increase of IL-6 expression (Pacheco et al., 2015), suggesting that overexpression of IL-6 might be a possible mechanism favoring persistence of some viruses. Similarly, in HIV-infected individuals increased levels of IL-6 positively correlated with levels of residual viremia, while in ectocervical tissues, the presence of IL-6 was correlated with enhanced transcriptional levels of HIV-1 (Rollenhagen and Asin, 2011).

In conclusion, there is plentiful evidence supporting a significant role of IL-6 during viral infections. However, certain scenarios create disparity of IL-6 production that may be detrimental to the cellular immune response during viral infections. Two different hypotheses may be considered to explain the change in IL-6 production during the immune response to viral infection: (i) the increased ability of some viral strains to overcome the immune response using a variety of evasion strategies (Beachboard and Horner, 2016), and consequently up-regulate the production of IL-6 as a result of increased viral loads, and (ii) polymorphisms in the IL-6 gene promoter stimulating overexpression of IL-6 during the immune response, a fact that has been shown to correlate with HBV progression (Lan et al., 2015). This last hypothesis might explain clinical reports correlating IL-6 overexpression with exacerbation of clinical outcomes in a sub-group of individuals during an outbreak caused by a single virus strain. Interestingly, this is consistent with experimental evidence in transgenic IL-6 mice (discussed below).

Experimental evidence supports the observation that overexpression of IL-6 during the viral immune response might induce viral persistence by impairing the polarization and functionality of Th1 cells and the lytic capacity of CD8 T-cells through different mechanisms, leading to chronic infections (**Figure 1**). As a consequence of the constant antigen stimulation, CD8 T-cells become unresponsive and fail to develop into memory CD8 T-cells, a situation that limits viral clearance (Shin and Wherry, 2007; Bardhan et al., 2016). Increased levels of IL-6 might also exacerbate the immunopathology during chronic infections by increasing inflammation followed by cytokine secretion and cellular recruitment as described during autoimmune diseases (Srirangan and Choy, 2010). In fact, this condition of increased inflammation may be an advantage for some viruses by providing new cellular targets for subsequent viral infections (Pingen et al., 2016).

An interesting question arises regarding the potential of some viruses to selectively up-regulate IL-6 levels as a possible immune evasion strategy. Although right now there is no scientific evidence to support the causal relationship between IL-6 levels and virulence, it could open new lines of research considering the capability of other intracellular microorganisms, like *Mycobacterium tuberculosis*, to induce overexpression of IL-6 to inhibit the autophagy process in infected cells (Dutta et al., 2012)

Further work is necessary to clarify the exact role of IL-6 during virus infections and the potential role of this cytokine to be used as a biomarker of viral virulence. Additional work exploring the potential therapeutic use of blocking IL-6 or different products affected by its activity might provide insight into controlling persistent viral infections. Caution is warranted for these kinds of studies, considering the conflicting effects of this interleukin during the progression of different viral infections. It is possible that the apparent contradictory function of IL-6 may depend on diverse triggering events that can be directly linked to the characteristics inherent to each virus infection. The pleiotropism of IL-6 function might stem from different viral stimuli activating distinct patterns of effector host mechanisms and their corresponding consequences.

AUTHOR CONTRIBUTIONS

LV-S, AV-R, LR, and MB conceived, designed, and wrote this manuscript.

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CAPÍTULO 8

El papel de la interleucina 6 durante las infecciones virales.

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Validation of a site-specific recombination cloning technique for the rapid development of a full-length cDNA clone of a virulent field strain of vesicular stomatitis New Jersey virus

Lauro Velazquez-Salinas[a,b,c], Steven J. Pauszek[a], Jose Barrera[d], Benjamin A. Clark[c], Manuel V. Borca[a], Antonio Verdugo-Rodriguez[b], Carolina Stenfeldt[a], Jonathan Arzt[a], Luis L. Rodriguez[a,*]

[a] Foreign Animal Disease Research Unit, USDA/ARS Plum Island Animal Disease Center, PO Box 848, Greenport NY 11944, USA
[b] National Autonomous University of Mexico, College of Veterinary Medicine and Animal Science, Mexico
[c] Oak Ridge Institute for Science and Education, PIADC Research Participation Program, Oak Ridge, TN, USA
[d] Leidos, Plum Island Animal Disease Center, P.O. Box 848, Greenport NY 11944, USA

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ABSTRACT

This study reports the use of a site-specific recombination cloning technique for rapid development of a full-length cDNA clone that can produce infectious vesicular stomatitis New Jersey virus (VSNJV). The full-length genome of the epidemic VSNJV NJ0612NME6 strain was amplified in four overlapping cDNA fragments which were linked together and cloned into a vector plasmid by site-specific recombination. Furthermore, to derive infectious virus, three supporting plasmid vectors containing either the nucleoprotein (N), phosphoprotein (P) or polymerase (L) genes were constructed using the same cloning methodology. Recovery of recombinant VSNJV was achieved after transfecting all four vectors on into BSR-T7/5 cells, a BHK-derived cell line stably expressing T7 RNA polymerase (PMID: 9847328). *In vitro* characterization of recombinant and parental viruses revealed similar growth kinetics and plaque morphologies. Furthermore, experimental infection of pigs with the recombinant virus resulted in severe vesicular stomatitis with clinical signs similar to those previously reported for the parental field strain. These results validate the use of site-directed specific recombination cloning as a useful alternative method for rapid construction of stable full-length cDNA clones from vesicular stomatitis field strains. The approach reported herein contributes to the improvement of previously published methodologies for the development of full-length cDNA clones of this relevant virus.

Vesicular stomatitis virus (VSV) has long been used in laboratories as a model to study the evolution of RNA viruses (Zarate and Novella, 2004; Novella et al., 2010: Velazquez-Salinas et al., 2016), as a vaccine platform for the expression of foreign proteins (Matassov et al., 2015), and as a prototype oncolytic virus for treatment of cancer in humans and dogs (Naik and Russell, 2009; LeBlanc et al., 2013; Velazquez-Salinas et al., 2017). In the field, VSV epidemic strains sporadically emerge causing economically significant outbreaks in livestock (Rodriguez, 2002). Currently, intrinsic determinants of virulence in epidemic VSV strains are the subject of experimental research (Velazquez-Salinas et al., 2018a, 2018b), and the ability to conduct rapid genetic manipulation of these strains is a key to understanding associated biological features.

VSV is an ~11 kb non-segmented negative-strand RNA virus and the prototype of the family Rhabdoviridae and the genus Vesiculovirus. VSV encodes five structural proteins: nucleocapsid (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and the large RNA-dependent RNA polymerase (L) (Wagner and Rose, 1996). The methodology of developing full-length cDNA VSV clones, involves the construction of four plasmid vectors (Whelan et al., 1995; Lawson et al., 1995; Harty et al., 2001). In applying this technique, one plasmid vector containing the full-length cDNA genome of VSV is used to drive the synthesis of a genome-length negative-sense transcript, while three supporting plasmids contain the components of the viral replication complex (the N, P and L genes). However, this approach typically requires multiple costly and time-consuming steps of cloning and sub-cloning because of the reliance on natural restriction sites in the viral genome.

Recently, recombination-based cloning methods have dramatically

* Corresponding author.
E-mail address: luis.rodriguez@ars.usda.gov (L.L. Rodriguez).

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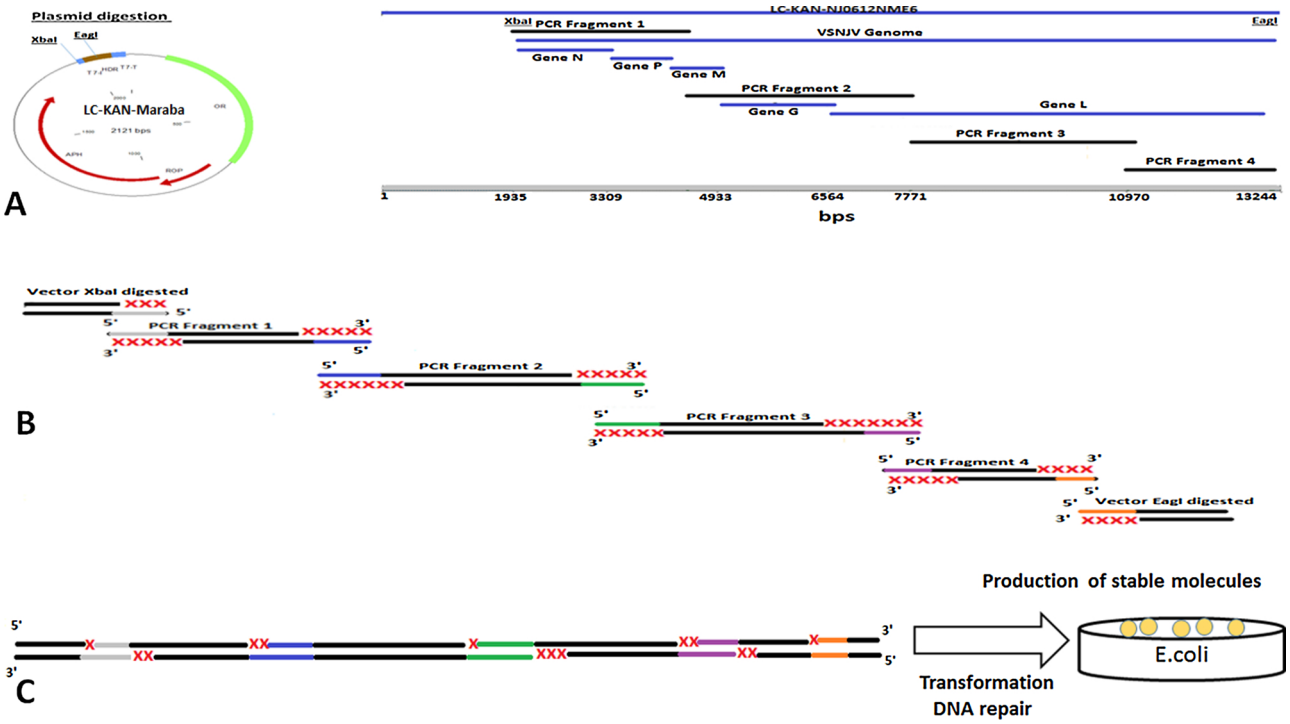

Fig. 1. Site-specific recombination cloning strategy to produce LC-Kan-NJ0612NME6. A) LC-Kan-Maraba plasmid was double digested, while the full-length genome of VSV was PCR amplified in four overlapping fragments. B) To promote site-specific recombination, plasmids and PCR fragments were incubated in the presence of VVpol. The 3′-5′ exonuclease activity of VVpol removes nucleotides at the 3′ends (represented with red X) of different overlapping fragments, promoting single-stranded alignment of complementary sequences (represented with similar colors). C) The final metastable non-covalently recombinant molecules of LC-Kan-NJ0612NME6 were produced. In theory, multiple nucleotide gaps or short overhangs (represented as red X) might be expected after the recombination reaction. Metastable molecules were repaired after being transformed into competent *Escherichia coli* (Hamilton et al., 2007; Irwin et al., 2012).

simplified the construction of full-length cDNA clones, mainly because of their lack of dependence on unique restriction sites in the target sequence, thus saving multiple steps of cloning and sub-cloning (Marsischky and LaBaer, 2004; Zhu et al., 2007). These methods have been used for the successful development of full-length cDNA clone systems for some important human and animal RNA viruses including dengue virus (Gibson assembly method), the porcine reproductive respiratory syndrome virus (Gibson assembly method), classical swine fever virus (In-fusion assembly method) and rabies virus (linear to-linear RedE/T recombination method) (Siridechadilok et al., 2013; Suhardiman et al., 2015; Kamboj et al., 2015; Nolden et al., 2016). The availability of novel recombination cloning methodologies, as well as the necessity to quickly and efficiently manipulate the genome of important RNA viral pathogens, encourages the continued improvement and validation of these methods.

This communication reports the validation of the In-fusion assembly method for the rapid development of a full-length cDNA clone derived from a highly virulent field strain of vesicular stomatitis New Jersey virus (VSNJV). This method is based on site-specific recombination catalyzed by the vaccinia virus DNA polymerase (VVpol) (Irwin et al., 2012).

The epidemic NJ0612NME6 strain was used as parental virus to derive the full-length cDNA clone. This highly virulent strain (GenBank accession #MG552609, Velazquez-Salinas et al., 2018a) was isolated from an epithelial lesion of a naturally infected equine in New Mexico during the 2012 VSNJV outbreak in the U.S (Velazquez-Salinas et al., 2014). To support the construction of different vector plasmids, multiple overlapping PCR products were synthesized (supplementary file 1). Total RNA was extracted from a high titer viral stock (HTVS) (titer $1 \times 10^{9.8}$ $TCID_{50}$/ml) using the RNeasy Mini Kit (QIAGEN). cDNA was synthesized by reverse transcription using random hexamers (Invitrogen) and SuperScript ™ II RNase H-Reverse Transcriptase (Invitrogen) following the manufacturer's instructions.

PCR's were conducted using the Platinum™ Hot Start PCR Mater mix (2X) kit in a final volume of 25 µl following manufacturer specifications using multiple sets of primers based on the NJ0612NME6 genomic sequence. Reactions were gel purified using ZYMORESEARCH DNA™ & Clean Concentrator ™ following manufacturer recommendations. PCR products were resuspended in a final volume of 6 µl in DNase/RNase-free Distilled Water. The overlapping PCR products were cloned by site-specific recombination using the VVpol (In-Fusion HD Cloning plus Kit, TaKaRa) into a previously described LC-KAN vector (Lucigen, Middleton, WI) (PMID: 20551913). Plasmids expressing the N, P or L proteins were derived from the pTIT vector as previously described (Finke and Conzelmann, 1999).

LC-Kan-Maraba and pTIT plasmids were doubled linear digested using XbaI- EagI-HF, and EcoRI-HF- BlpI respectively. Digestions were gel purified (LC-Kan-Maraba = ˜2057 bp, and pTIT = ˜3056 bp) as described above, and resuspended in DNase/RNase-free Distilled Water.

Specific reactions for the construction of each plasmid can be found in supplementary file 1. Fig. 1 illustrates the recombination cloning strategy used in this study.

The identification of stable vector plasmids was done by evaluating individual colonies of each reaction. Colonies were inoculated in 5 mL of 2x YT (Yeast Extract Tryptone) (TEKNOVA) containing 50 µg/mL of Kanamycin (LC-Kan-VSNJV plasmid), or Carbenicillin (pTIT plasmids) and incubated at 37 °C overnight while shaking. Plasmids were purified using the QIAprep kit (QIAGEN) following the manufacturer instructions. Plasmids containing correct size inserts were identified by restriction endonuclease analysis and sequenced to confirm fidelity to the wild type sequence. Selected cultures were used to inoculate 250 mL in 2x YT medium as described above. Plasmids were purified using the QIAGEN Plasmid Maxi Kit and re-sequenced. Sequencing reactions were conducted using multiple sets of primers as previously described (Pauszek and Rodriguez, 2012).

As a result of the recombination reactions, four stable plasmids were

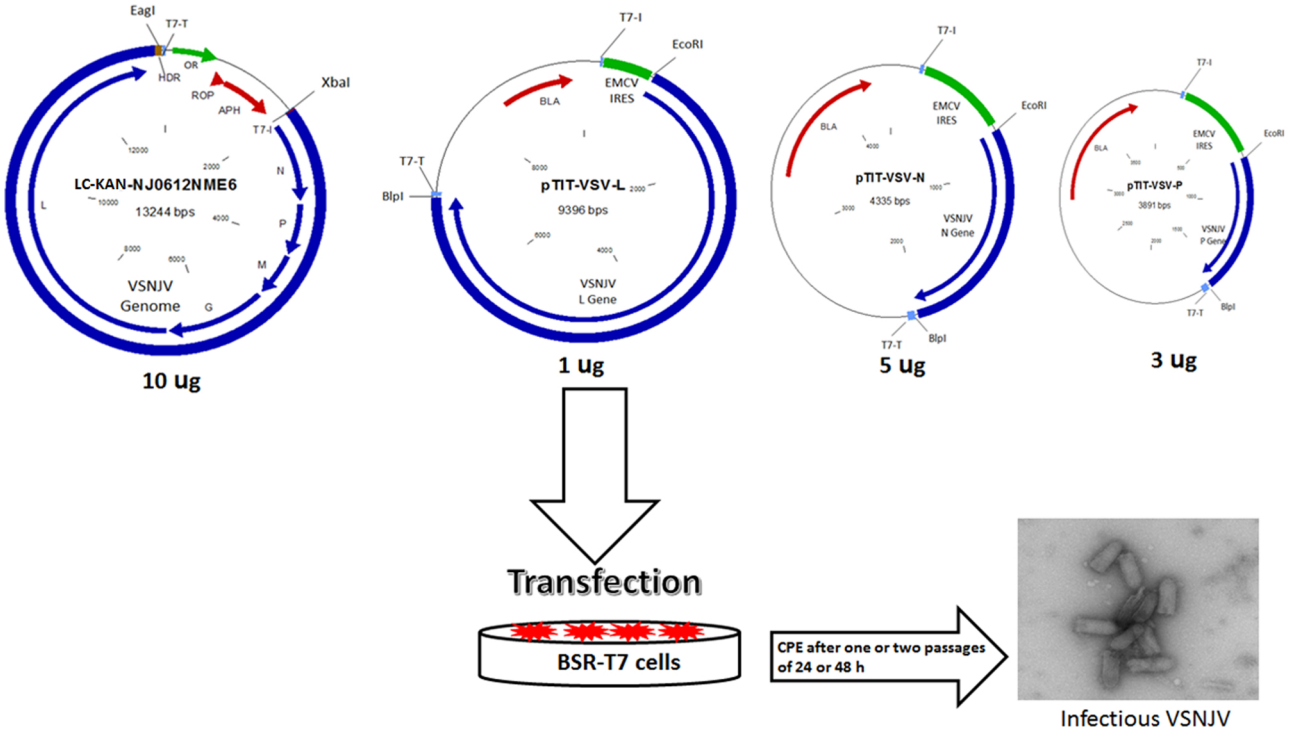

Fig. 2. Recovery of rNJ0612NME6. Specific concentrations of developed plasmids containing the full-length genome of VSV (LC-Kan-NJ0612NME6), and individual genes N (pTIT-VSNJV-N), P (pTIT-VSNJV-P), and L (pTIT- VSNJV-L) were used to transfect BSR-T7/5 cells at 75–80% confluency using a calcium phosphate transfection kit (ThermoFisher). After an overnight incubation, cells were split into T25 flasks, and typical CPE produced by VSV was observed after 24–48 hrs. Plasmid features include: restriction sites (EagI, XbaI, EcoR1, and BlpI), T7 promoter (T7-I), T7 terminator (T7-T), replication origin (OR), gen ROP, E.coli regulatory protein (ROP), kanamycin resistance gene (APH), ampicillin resitance gene (BLA), hepatitis delta ribozyme (HDR), and encephalomyocarditis virus internal ribosome entry site (EMCV IRES).

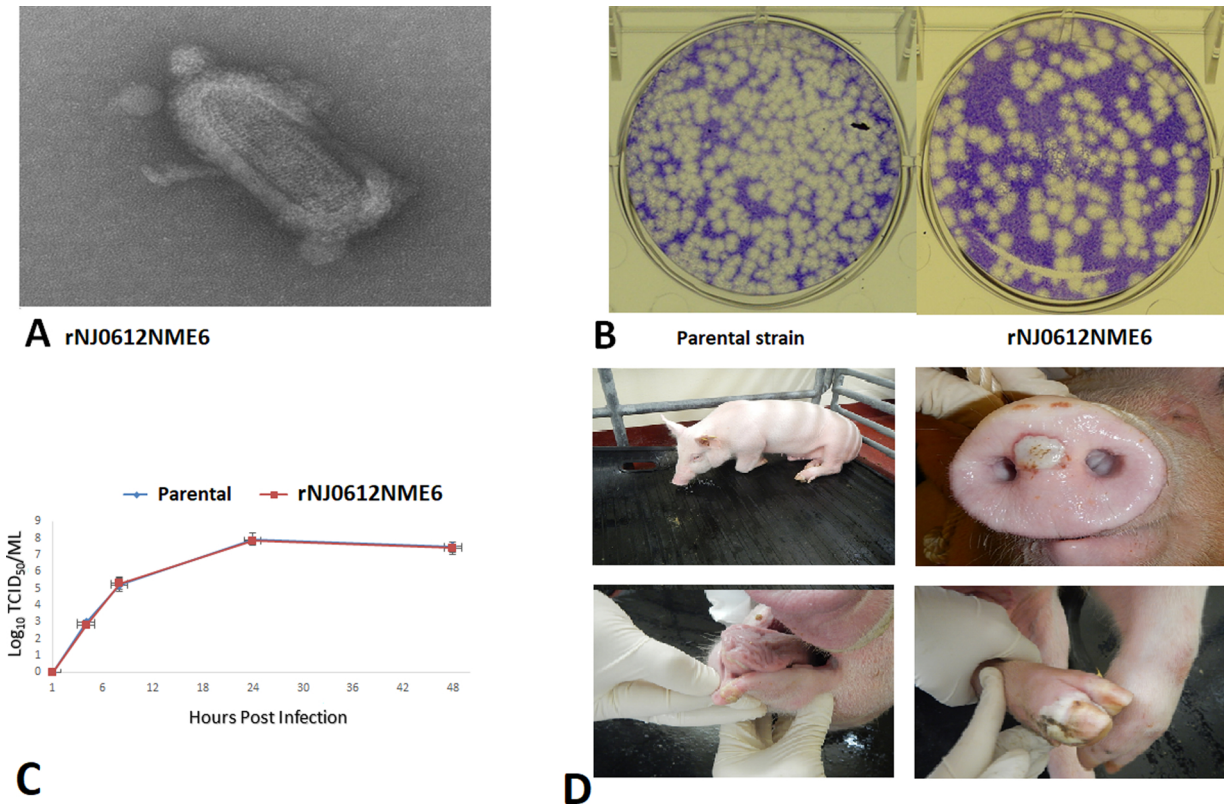

Fig. 3. *In vitro* and *in vivo* characterization of rNJ0612NME6. A) rNJ0612NME6 confirmed the typical rhabdovirus bullet shape when purified and visualized by electronic microscopy. B) Infection of BHK-21 cells with rNJ0612NME6 and the parental wild-type virus confirmed similar plaque formation, and growth kinetics (C). D) Intradermal inoculation of the snout skin of four pigs with 1×10^7 $TCID_{50}$ of rNJ0612NME6 resulted in the classical VSV clinical features including: depression, fever, vesicle development at the inoculation site, and systemic infection evidenced by the presence of epithelial lesions at secondary sites (lips and feet). Nasal and oral swabs analyzed by real time RT-PCR and viral isolation confirmed the presence of rNJ0612NME6.

obtained: LC-Kan- NJ0612NME6, pTIT-VSNJV-N, pTIT-VSNJV-P, and pTIT-VSNJV-L (Fig. 2). The recovery of recombinant VSNJV (rNJ0612NME6) was achieved in a vaccinia virus free system using BSR-T7/5 cells as previously described (Buchholz et al., 1999; Harty et al., 2001). More details about this process are shown in Fig. 2, and supplementary file 2.

The presence of rNJ0612NME6 in the supernatant of transfected BSR-T7/5 cells was confirmed by real time PCR conducted as previously described (Velazquez-Salinas et al., 2018b). The extracted RNA was treated with 2 units of DNAseI (BioLabs) to remove any residual/contaminating LC-Kan-NJ0612NME6. Additionally, the infection of rNJ0612NME6 in Vero cells was neutralized with a pool of polyclonal antisera collected during the experimental infection of pigs with the parental strain (Velazquez-Salinas et al., 2018b). Furthermore, full-length genomic sequence analysis confirmed 100% sequence identity between rNJ0612NME6 and the parental virus.

Finally, to assess the phenotypic characteristic of rNJ0612NME6, in *vitro* and *in vivo* experiments were conducted, including the infection of pigs, a natural host of VSNJV. Experimental infections of pigs were conducted under an experimental protocol approved by the institutional animal care and use committee (IACUC protocol #245-05-14R) in a biosafety level 3 (BSL-3Ag) facility at the United Sates Department of Agriculture's Plum Island Animal Disease Center (PIADC). The results of these experiments confirmed similar phenotypic characteristics of rNJ0612NME6 and the parental virus both *in-vitro* and *in-vivo* (Fig. 3). Additional information about the methodology used to perform these experiments is summarized in supplementary file 2.

In conclusion, the aim of this study was to validate the efficiency of the site-specific recombination approach based on the Infusion assembly method for the rapid development of a full-length cDNA clone derived from a highly virulent field strain of VSNJV. The results obtained in this study concur with a previous publication utilizing a similar approach with classical swine fever virus (Kamboj et al., 2015), supporting the efficiency of this methodology for the rapid and efficient assembly of vector plasmids. Overall, the cloning process was slightly more efficient for the supporting pTIT vector plasmids than for LC-Kan-NJ0612NME6 which contains multiple fragments. The results of this study will contribute to the improvement of the previously described methodology for developing full-length cDNA clones of VSV (Whelan et al., 1995; Lawson et al., 1995) by excluding multiple sub-cloning steps, thereby allowing for rapid genetic manipulation of new emerging VSV strains using a vaccinia virus-free system.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.01.003.

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CAPÍTULO 9

La mutación M51R en la proteína de matriz del VSNJV cepa NJ0612NME6 afecta la habilidad de esta cepa para replicar en cultivos de macrófagos porcinos, resultando en una atenuación significativa en el cerdo.

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A Single Amino Acid Substitution in the Matrix Protein (M51R) of Vesicular Stomatitis New Jersey Virus Impairs Replication in Cultured Porcine Macrophages and Results in Significant Attenuation in Pigs

*Lauro Velazquez-Salinas[1,2,3], Steven J. Pauszek[1], Lauren G. Holinka[1], Douglas P. Gladue[1], Steven I. Rekant[1,3], Elizabeth A. Bishop[1], Carolina Stenfeldt[1,4], Antonio Verdugo-Rodriguez[2], Manuel V. Borca[1], Jonathan Arzt[1] and Luis L. Rodriguez[1]**

[1]Foreign Animal Disease Research Unit, USDA/ARS Plum Island Animal Disease Center, Greenport, NY, United States, [2]College of Veterinary Medicine and Animal Science, National Autonomous University of Mexico, Mexico City, Mexico, [3]PIADC Research Participation Program, Oak Ridge Institute for Science and Education, Oak Ridge, TN, United States, [4]Department of Veterinary Population Medicine, University of Minnesota, St. Paul, MN, United States

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Edited by:
Juan-Carlos Saiz, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Spain

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Maria Teresa Sanchez-Aparicio, Icahn School of Medicine at Mount Sinai, United States

***Correspondence:**
Luis L. Rodriguez
luis.rodriguez@usda.gov

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In this study, we explore the virulence of vesicular stomatitis New Jersey virus (VSNJV) in pigs and its potential relationship with the virus's ability to modulate innate responses. For this purpose, we developed a mutant of the highly virulent strain NJ0612NME6, containing a single amino acid substitution in the matrix protein (M51R). The M51R mutant of NJ0612NME6 was unable to suppress the transcription of genes associated with the innate immune response both in primary fetal porcine kidney cells and porcine primary macrophage cultures. Impaired viral growth was observed only in porcine macrophage cultures, indicating that the M51 residue is required for efficient replication of VSNJV in these cells. Furthermore, when inoculated in pigs by intradermal scarification of the snout, M51R infection was characterized by decreased clinical signs including reduced fever and development of less and smaller secondary vesicular lesions. Pigs infected with M51R had decreased levels of viral shedding and absence of RNAemia compared to the parental virus. The ability of the mutant virus to infect pigs by direct contact remained intact, indicating that the M51R mutation resulted in a partially attenuated phenotype capable of causing primary lesions and transmitting to sentinel pigs. Collectively, our results show a positive correlation between the ability of VSNJV to counteract the innate immune response in swine macrophage cultures and the level of virulence in pigs, a natural host of this virus. More studies are encouraged to evaluate the interaction of VSNJV with macrophages and other components of the immune response in pigs.

Keywords: vesicular stomatitis, pathogenesis, M51R, virulence, macrophages, immune response, type 1 interferon

INTRODUCTION

Vesicular stomatitis virus (VSV) is an arbovirus, and the prototype for the viral family Rhabdoviridae and the *Vesiculovirus* genus. Two distinct serotypes of VSV have been identified and defined: Indiana (VSIV) and New Jersey (VSNJV) (Rodriguez, 2002; Velazquez-Salinas et al., 2016b). VSNJV is responsible for most of the vesicular disease cases reported annually in the Americas (Rodriguez, 2002; Mead et al., 2009; Velazquez-Salinas et al., 2014). Because of the clinical resemblance between vesicular stomatitis (VS) and foot-and-mouth disease, one of the most economically devastating diseases for the livestock industry, quarantines and trade embargoes are often imposed in VS-affected premises (Timoney, 2016).

The ~11 kb RNA genome of VSV encodes five structural proteins: nucleocapsid (N), phosphoprotein (P), matrix (M), glycoprotein (G), and the large RNA-dependent RNA polymerase (L) (Dietzgen et al., 2011). The M protein is one of the best characterized proteins in the VSV proteome. Its functions are associated with apoptosis, virus assembly, budding, cytopathic effect, inhibition of host transcription, nucleocytoplasmic transport of host RNA, and inhibition of host interferon response (Ahmed et al., 2003; Rajani et al., 2012, Hastie et al., 2013; Melzer et al., 2017). In this context, the methionine residue at position 51 of the M protein plays a key role in maintaining optimal functionality of this protein. Substitution of this highly conserved residue for the amino acid arginine (M51R) results in profound loss of viral fitness by reducing the virus's ability to block innate immune responses, causing increased production of type 1 interferon (IFN) and induction of interferon stimulated genes (Lyles et al., 1996; Stojdl et al., 2003; Ahmed et al., 2008; Varble et al., 2016; Melzer et al., 2017). Possible mechanisms associated with the dysfunctionality of M51R phenotype might involve its inability to inhibit the activity of host RNA polymerases (Ahmed et al., 2008) and its interaction with protein complexes like the nucleoporin Nup98, and the export RNA factor Rae1 (Faria et al., 2005; Rajani et al., 2012; Quan et al., 2014).

It is well-documented that VSNJV causes sporadic vesicular disease outbreaks in livestock in the southwestern U.S. at 8–10-year intervals. Each outbreak cycle is associated to a distinct viral strain among many circulating in endemic regions of Mexico (Rodriguez et al., 2000, Rainwater-Lovett et al., 2007, Arroyo et al., 2011; Velazquez-Salinas et al., 2014). However, the intrinsic and extrinsic factors associated with the emergence and spread of these particular strains remain unclear.

Recently, we described that an epidemic strain (NJ0612NME6), responsible for the most extensive VS outbreak in the southwestern US's recent history, had an increased ability to disrupt innate immune responses in experimentally inoculated pigs, a natural host of this virus (Velazquez-Salinas et al., 2018a). Specifically, decreased levels of systemic type 1 IFN and tumor necrosis factor (TNF), along with increased levels of interleukin 6 were associated with more prolonged periods of higher fever, higher RNAemia, as well as an increased number of vesicular lesions in pigs infected with NJ0612NME6, when compared with a non-epidemic strain from southern Mexico (NJ0806VCB).

In this study, we aimed to gain further insight about the role of innate immune responses during the pathogenesis of VSNJV in pigs. For this purpose, we genetically engineered a cDNA clone of the highly virulent strain of VSNJV (NJ0612NME6) to introduce a single amino acid mutation in the M protein at position 51 to develop a mutant virus (M51R). For *in vitro* testing, we used both non-immune and immune cells. As non-immune cells, we used primary fetal porcine kidney cells that contain a mix of epithelial, fibroblasts, and endothelial-like cell phenotypes (Richter et al., 2012). Since skin and mucosal surfaces are considered to be the primary anatomical targets during the natural infection in livestock (Rodriguez et al., 2000), we considered this cellular mix appropriate to evaluate the innate immune response. As immune cells we used primary monocyte-derived macrophages. These cells have been successfully used to characterize the innate cellular response to infection by other viruses including African swine fever virus, classical swine fever, and VSV (Carlson et al., 2016; Velazquez-Salinas et al., 2016a, 2018b). Macrophages have been shown to play a preponderant role in the virulence of VSV in mice (Ciavarra et al., 2005; Junt et al., 2007; Simon et al., 2010). Additionally, we conducted a comprehensive *in-vivo* pathogenesis study using a previously described pig infection model (Martinez et al., 2003; Velazquez-Salinas et al., 2017, 2018b). The findings of this study are discussed in the context of the role that innate immune responses might play in determining the virulence of VSNJV in pigs.

MATERIALS AND METHODS

Viral Strains

A high-titer virus stock ($1 \times 10^{9.8}$ $TCID_{50}$/ml) of the highly virulent VSNJV NJ0612NME6 strain grown in baby hamster kidney cells (BHK-21) was used to construct a cDNA clone by site-direct recombination using a reverse genetics system as previously described (Velazquez-Salinas et al., 2018b, 2019).

Cell Lines

Monkey kidney epithelial cells (Vero), BHK-21, Madin-Darby bovine kidney epithelial cells (MDBK-t/2), and primary fetal porcine kidney cell cultures (FPKC) were obtained from the Foreign Animal Disease Diagnostic Laboratory (FADDL) at the Plum Island Animal Disease Center (PIADC), Greenport, NY. Porcine monocytes-derived macrophages cell cultures (PM-MQC) were obtained as previously described (Zsak et al., 1996). The BSR-T7/5 cell line that constitutively expresses the T7 RNA polymerase (Buchholz et al., 1999) was used to recover the recombinant viruses.

For the purpose of this work, we considered stromal cells (epithelial and fibroblasts) like FPKC as non-immune cells, while that white blood cells that mediate innate immunity like PM-MQC where considered immune cells.

Construction of the M51R Mutant Virus

LC-KAN-NJ0612NME6 plasmid containing the full-length genome of the highly virulent VSNJV NJ0612NME6 strain was used as a template to obtain the plasmid LC-KAN-NJ0612NME6-M51R carrying the codon substitution ATG-AGA

at codon 51 of the matrix gene. This substitution was done using the QuickChange XL site-directed mutagenesis kit (Stratagene), following the manufacturer recommendations. For this purpose, the following set of primers was designed: M51R-F 5′-GATTTCTTCGGAAT GGAGGATAGAGACTTATATGAC AAGGACTCCT-3′ and M51R-R 5′-AGGAGTCCTTGTCA TATAAGTCTCTATCCTCCATTCCGAAGAAATC-3′.

To recover only the newly amplified plasmid NJ0612NME6-M51R, the site-directed mutagenesis reaction was digested with the type IIM restriction endonuclease Dpn1, and cloned into XL10-Gold ultra-competent cells.

In vitro Rescue of the rNJ0612NME6 and M51R Viruses

Viral rescue was achieved from a cDNA VSV clone as previously described (Velazquez-Salinas et al., 2019). Briefly, independent transfections were conducted in BSR-T7/5 cells, using either LC-KAN-NJ0612NME6 or LC-KAN-NJ0612NME6-M51R plasmids and supporting plasmids pTIT-VSNJV-N, pTIT-VSNJV-P, and pTIT-VSNJV-L. After viral recovery, high titer viral stocks (HTVS) of rNJ0612NME6 (parental virus) and M51R viruses were made in Vero cells. Sequence identity of both viruses was confirmed by sequencing analysis as previously described (Pauszek and Rodriguez, 2012).

Cell Viability Assay

To evaluate differences in the ability to induce cytotoxicity between rNJ0612NME6 and M51R viruses, we conducted the MTT assay. This assay is a rapid colorimetric technique for determining cellular growth and survival by assessing the enzymatic capability of the cell to reduce the tetrazolium dye MTT (Mosmann, 1983).

The MTT assay was conducted using the MTT Cell Proliferation Assay kit (ATCC bioproducts Cat#30-1010K) following the manufacturer instructions. Briefly, the MTT assay was conducted in 96-well plates using preformed monolayers of FPKC (~1×10^5 cells per well). Wells were infected in octuplicate with 100 µl of either rNJ0612NME6 or M51R viruses at different MOI's (10–0.00001). Twenty-four hours post-infection (hpi), 10 µl of MTT dye was added to each well and incubated for 2 h at 37°C. Finally, 100 µl of detergent reagent was added to each well and incubated for 2 h at room temperature. Plates were read in a microplate reader using an optical density of 570 nm. Optical values were converted to percentages of cell survival by comparing the values obtained from infected wells to the average value achieved by the negative control (DMEM). To confirm the ability of FPKC to mount an antiviral response, an additional set of plates was treated with 2 units/ml of recombinant porcine IFN-α2A per well 24 h prior to infection. Since the induction of apoptosis has been reported in different cell lines after treatment with IFN (Chawla-Sarkar et al., 2003), controls for this experiment included: FPKC treated only with IFN-α2A, and uninfected cells.

In vitro Growth Characterization

To assess the ability of rNJ0612NME6 and M51R viruses to grow in FPKC and PM-MQC cells, multistep growth curves were performed in triplicate at a MOI of 0.01 $TCID_{50}$. After 1 h of absorption (time zero), samples were collected at 1, 4, 8, 24, and 48 hpi. Viral titrations were conducted in BHK-21 cells by endpoint titration assay in 96-well plates as previously described (Velazquez-Salinas et al., 2018a). Final titers were determined by the Reed and Muench method and expressed as $TCID_{50}$/ml (Reed and Muench, 1938).

Transcriptional Immune Response

To evaluate the transcriptional immune profile associated with the *in vitro* infection of rNJ0612NME6 and M51R mutant viruses in FPKC and PM-MQC cells, a total of 42 pig genes were studied including: interferons type I (IFNα1, IFNα7-11, IFNα9, IFNα10, IFNα13, IFNα14, IFNα15, IFNα16, IFNα17, IFNβ1, IFNδ1, IFNδ2, IFNδ5-9-11, IFNδ6, IFNδ7, IFNδ8, IFNκ, IFNε, and IFNω1-6), type II (IFNγ) and type III (IFNλ1), interferon regulatory factors (IRF 1, IRF 3, IRF 6, IRF 7, and IRF 9), transcription factors (SAT 1 and SAT 2), interferon stimulated genes (ISG) (IFIT1, IFIT2, RIG-I, MX1, MX2, OAS1, GBP1, BST2, PKR, ISG20, and TRIM25), and chemokines and cytokines (IL-6, TNF-α, and CXCL10). Cells were infected at a MOI of 10 $TCID_{50}$ and total cellular RNA was extracted after 5 hpi using the RNeasy Mini Kit (QIAGEN) following the manufacturer's instructions. Analyses were conducted by quantitative reverse transcription real-time polymerase chain reaction (qRT-PCR) as previously described (Borca et al., 2008). A difference of at least threefold either up or down between normalized mRNA expression levels in VSV-infected cells and mock-infected cells was considered as significant (Brukman and Enquist, 2006; Borca et al., 2008).

Plaque Assay

To evaluate the plaque morphology on FPKC produced by rNJ0612NME6 and M51R viruses, we conducted a standard plaque assay. Preformed monolayers of FPKC on six-well plates were infected with each virus, and incubated for 1 h at 37°C. Cells were then overlaid with tragacanth gum (0.6%) and incubated for 48 h at 37°C. Finally, cells were stained with crystal violet to make evident the plaque formation in infected cells.

Animal Experiments

To compare the virulence and transmissibility of rNJ0612NME6 and M51R, two experiments (one for each virus) were conducted in pigs, a natural host of VSV, as previously described (Martinez et al., 2003; Velazquez-Salinas et al., 2017, 2018a). For each experiment, eight male, 8–10 weeks old Yorkshire pigs (~30 kg weight) were randomly segregated into two groups of four (directly inoculated and contact) and separated by double fencing to prevent direct contact. After 1 week of acclimation, pigs in the inoculated group were sedated using a mixture of xylazine, ketamine, and Telazol (4, 8, and 3 mg/kg, respectively) and infected by intradermal inoculation by scarification in the snout with a dose of 10^7 $TCID_{50}$ of virus in 50 µl of either rNJ0612NME6 or M51R. Twenty-four hours after infection, inoculated pigs were allowed to co-mingle with pigs from their respective contact group until the end of the experiment (day 21).

Ethics Statement

Animal experiments were performed under biosafety level 3AG conditions in the animal facilities at PIADC. All experimental procedures were carried out in compliance with the Animal Welfare Act (AWA), the 2011 Guide for Care and Use of Laboratory Animals, the 2002 PHS Policy for the Humane Care and Use of Laboratory Animals, and U.S. Government Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training (U.S. Office of Science and Technology Policy, 1985), as well as specific animal protocols reviewed and approved by the PIADC Institutional Animal Care and Use Committee of the US Departments of Agriculture and Homeland Security (protocol number #245-05-14R).

Clinical Evaluation and Sampling

Clinical evaluation and sample collection were conducted daily in all animals from 0 to 10 days and at 14 and 21 days post-infection (dpi). Clinical evaluation included measurement of rectal temperatures and assessment of vesicular lesion development using a cumulative clinical scoring system based on the number and location of lesions as previously described (Velazquez-Salinas et al., 2018a). Briefly, maximum scores of 45 and 46 were allowed for direct inoculated and contact pigs, respectively. A characteristic lesion in each of the 16 digits contributed to the cumulative score with two points, while one or two points are assigned for lesions on the snout of directly inoculated and contact animals correspondingly, and two points added for lesions in carpal/tarsal skin, oral cavity, and lower lip. Sample collection included: whole blood, serum, oropharyngeal (OP) swabs, and nasal swabs. After collection, samples were processed as previously described (Velazquez-Salinas et al., 2018a) and stored at −70°C until testing.

Post-mortem Sample Collection

To compare the biodistribution between rNJ0612NME6 and M51R, the two pigs displaying the highest clinical scores from each of the two groups (inoculated and contact) from each of the two experiments were chosen for tissue harvest at 21 dpi. Pigs were deeply sedated using an intramuscular injection of Telazol, ketamine, and xylazine at 4.5, 12, and 6 mg/kg, respectively, and posteriorly were humanely euthanized by exsanguination. Tissue collection included: tonsil of the soft palate, submandibular lymph node, spleen, snout skin, popliteal lymph node, and skeletal muscle. Duplicate aliquots of each sample containing 30 mg of each tissue were prepared and frozen at −70°C until processing.

Viral RNA Detection

To contrast the viral growth dynamics during infection in pigs between rNJ0612NME6 and M51R, the presence of viral RNA extracted from different biological samples collected during the time course of the experiment was assessed by real-time RT-PCR (rRT-PCR). RNA extraction was conducted using the Ambion's MagMax-96 Viral RNA Isolation Kit (Ambion, Austin, TX) as previously described (Arzt et al., 2010). rRT-PCR targeting the VSNJV nucleocapsid gene (N) was conducted as previously described (Scherer et al., 2007) but using a different forward primer (5′-GCACTTCCTGATGGGAAATCA-3′) to match the native sequence of the NJ0612NME6 viral strain (Velazquez-Salinas et al., 2018b). rRT-PCR was carried out using 2.5 µl of RNA on an ABI 7500 system (Applied Biosystems, Austin, TX). Results were expressed as RNA genome copy numbers per 2.5 µl of RNA.

Virus Isolation

The detection of infectious virus in biological samples collected during animal experiments was conducted in BHK-21 cells. To eliminate potential bacterial contamination, samples were filtered through 0.45 µm Spin-X filter columns (Costar cat. No 8163). Afterwards, samples were diluted 1:5 in cell culture media and 500 µl of each dilution was used to overlay preformed BHK-21 cell monolayers in 24 well-plates. After 1 h of incubation at 37°C, 2 ml of maintenance media was added to each well and monitored for cytopathic effect (CPE) for 72 h. Positive samples were confirmed by rRT-PCR and titrated by endpoint titration assay in 96 well-plates as described in the previous sections.

Detection of Systemic Type I IFN in Pigs

To contrast the systemic type I IFN induction between rNJ0612NME6 and M51R during experimental infection in pigs, serum samples collected during the acute stage of the infection were assessed by Mx-CAT reporter assay (Fray et al., 2001; Francois et al., 2005). Analyses were conducted on MDBK-t/2 cells using a CAT ELISA kit (Roche Applied Sciences, Indianapolis IN) as previously described (Perez-Martin et al., 2012, Fernandez-Sainz et al., 2015). A standard curve was created using recombinant IFN-α2A at concentrations from 1.95 to 1,000 U/ml and used to determine international units of antiviral activity per ml for each sample. The presence of type1 IFN in serum samples is positively correlated to the induction of the Mx gene in MDBK-t/2. This correlation has been previously validated (Francois et al., 2005), and the Mx-CAT reporter assay has been successfully used in previous experiments to analyze the presence of type I IFN in pigs (Fernandez-Sainz et al., 2015; Velazquez-Salinas et al., 2018a).

Serum Neutralization Assay

The development of neutralizing antibodies against VSNJV during the infection of pigs with either rNJ0612NME6 or M51R was monitored by serum neutralization assay in Vero cells (96-well plates containing 1×10^6 cells per plate), and using 1,000 $TCID_{50}$ of VSNJV as previously described (Flanagan et al., 2001). Serum neutralizing activity was reported as the reciprocal of the highest dilution giving 100% inhibition of CPE.

Statistical Analysis

Statistical significance between groups was determined using the Holm-Sidak method, with alpha = 0.05. Calculations were performed using GraphPad Prism version 8.01 for Windows (GraphPad Software, La Jolla, California, USA, www.graphpad.com).

RESULTS

To gain a better understanding of the role of innate immune responses during the replication of VSNJV, FPKC (non-immune cells) and PM-MQC (immune cells) were used. When evaluated in FPKC cells, no differences were observed between rNJ0612NME6 and M51R in viral capacity to induce cytotoxicity at different MOI (**Figure 1A**). Conversely, 100% cell survival was recorded during the infection with either virus at any MOI when cells were pre-treated with an external source of type I IFN, demonstrating the competence of FPKC in responding to type I IFN stimulation (**Figure 1A**). Although M51R grew to slightly lower titer than rNJ0612NME6, no significant differences in the viral growth kinetics were found between viruses (**Figure 1B**).

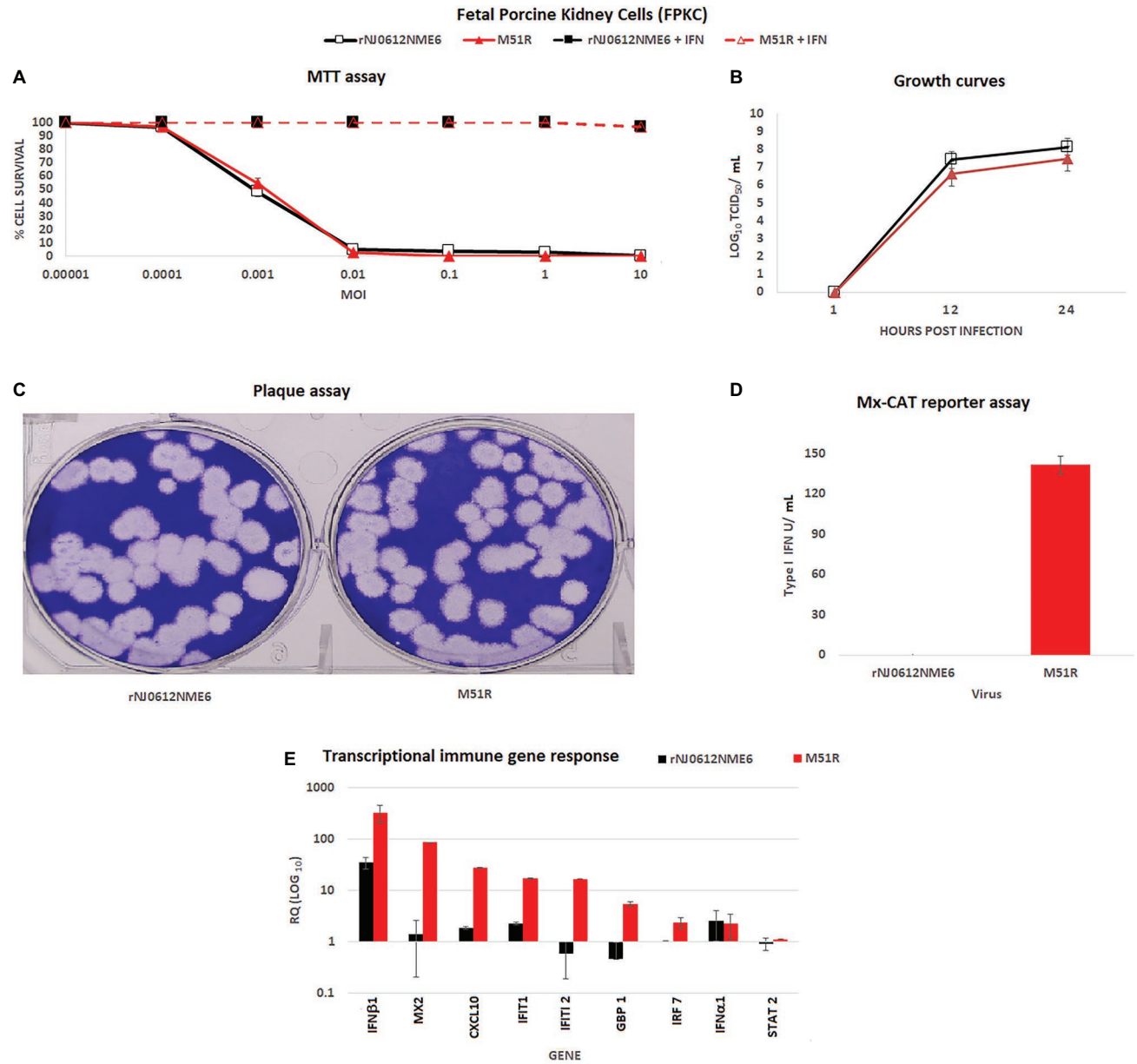

FIGURE 1 | *In vitro* characterization of rNJ0612NME6 and M51R viruses in fetal porcine kidney cells (FPKC). **(A)** MTT assay was used to assess differences between both viruses to induce cytotoxicity in FPKC at different MOI's, using untreated and previously treated cells with an external source of type I interferon (IFN) (2 units/ml of recombinant porcine IFN-α2A per well). Apoptosis was not detected in both controls: FPKC only treated with IFN-α2A, and untreated ones. **(B)** Multistep growth curves were performed in FPKC to compare virus yields between both viruses at specific times post-infection, using at initial MOI = 0.01. **(C)** Conventional plaque assay was conducted on FPKC to monitor differences in plaque formation between both viruses. **(D)** Mx-CAT reporter assay was used to test the ability of both viruses to induce the production of type I INF in FPKC. Samples were collected from multistep growth curves experiments at 24 hours post-infection (hpi). **(E)** Differences in the transcriptional immune gene response induced by different viruses on FPKC, were assessed by qRT-PCR. RQ values represent relative quantities of mRNA accumulation (estimated by $2^{-\Delta\Delta Ct}$) with their corresponding SD. All experiments were conducted in triplicates.

Overall, both viruses produced similar plaque size and morphology (**Figure 1C**). However, a strong type I IFN response was detected only in M51R-infected cells (**Figure 1D**). The increased innate response in M51R infected cells was further confirmed by transcriptional analysis performed at 5 hpi. M51R induced significantly higher mRNA levels of IFNβ1, Mx2, CXCL10, IFT1, IFIT2 GBP1, and IRF7 compared to cells infected with rNJ0612NME6 (**Figure 1E**). Also, no significant differences were observed in the transcriptional levels of IFNα1 and STAT2 indicating that they were not induced by either of the two viruses.

In contrast, infection of PM-MQC using an MOI 0.01 revealed a significantly decreased capacity of M51R (~3 log reduction; $p < 0.05$) to grow in PM-MQC when compared with rNJ0612NME6 virus (**Figure 2A**). To analyze the transcriptional immune response, PM-MQC were infected with each virus at a MOI of 10 and samples were collected at 5 hpi and evaluated by qRT-PCR (**Figures 2B–E**). Infection with M51R resulted in a significant ($p < 0.05$) increase of mRNAs associated with type 1 IFNs including IFNβ1 and different subtypes of IFNα, as well as type II IFN (IFNγ). Conversely, rNJO0612NM6 was able to significantly ($p < 0.05$) decrease the transcriptional levels of IFNα9 and IFNα15 genes. No differences were observed in the transcriptional levels of type II IFN and other subtypes of type I IFN genes (**Figure 2B**).

Compared with rNJ0612NME6, infection with M51R resulted in a significant ($p < 0.05$) increase in mRNA for several cytokines and chemokines (i.e., IL-6, TNF, and CXCL10) (**Figure 2C**), interferon regulatory factors and transcription factors (IRF7, IRF9, STAT1, and STAT2) (**Figure 2D**), and ISG (IFIT1, IFIT2, MX1, MX2, OAS1, GBP1, BST2, and PKR) (**Figure 2E**).

Virulence in Pigs

We compared the virulence between rNJ0612NME6 and M51R using a previously established model to assess the pathogenesis of VSV in pigs. The experimental design allowed not only evaluation of clinical signs (fever and vesicular lesions), viral shedding, RNAemia, and antibody response in directly inoculated and contact infected animals, but also assessed the ability of each virus to be transmitted by direct contact.

Clinical Assessment

Rectal temperatures $\geq$39.8°C occurred in all pigs directly inoculated and contact-infected with rNJ0612NME6 at 3–7 dpi or days post exposure (dpe) (**Figure 3A**). Conversely, none of the pigs directly inoculated with M51R or those in direct contact had fever throughout the course of the experiment.

The development of vesicular lesions was monitored daily using the scoring system described above. Significantly higher clinical scores ($p < 0.05$) were observed in pigs infected with rNJ0612NME6 (directly inoculated: 13.5 ± 1.9 and contact exposure: 16.5 ± 1.2) than pigs infected with M51R (directly inoculated: 1.2 ± 0.4 and contact exposure: 4.0 ± 1.1) (**Figure 3B**).

Direct inoculation with M51R resulted in the development of small vesicular lesions at the site of inoculation with the exception of pig # 38, where the lesion extended outside the inoculation site (**Figure 4A**). In this animal, vesicular lesions developed at 2 dpi increased in size and ruptured between 3 and 5 dpi. No secondary lesions were observed, except in one animal, where two small vesicles were detected in the carpal region of the front limbs at 7 dpi. Although M51R was transmitted by contact exposure, vesicular lesions were small and appeared only in the carpal region of the back limbs of two of the four exposed pigs between 6 and 7 dpe (**Figure 4A**). These small lesions were confirmed positive for VSV by rRT-PCR (not shown).

No signs of obtundation or lameness were observed in any of the M51R infected animals throughout the course of the experiment.

Conversely, direct inoculation of pigs with rNJ0612NME6 resulted in the appearance of vesicular lesions at the inoculation sites at 2 dpi. Lesions increased in size considerably and ruptured around 3 and 5 dpi (**Figure 4B**). Vesicular lesions appeared at secondary sites starting at 3 dpi. All contact exposed animals developed lesions on the snout, lips, tongue, and feet (coronary bands, tarsal, and carpal regions) by 3 dpe (**Figure 4B**). The extensive size of the vesicular lesions on the feet of these animals was associated with severe lameness after 5 dpi or dpe and severe obtundation in all animals during the febrile period.

Viral Shedding

The extent and duration of viral shedding in pigs was evaluated by the collection of daily nasal and oral swabs which were analyzed by rRT-PCR and viral isolation.

There were significant differences ($p < 0.05$) between the two viruses both in the extent and duration of nasal shedding measured by rRT-PCR with rNJ0612NME6 shedding at higher levels and longer periods than M51R. While levels of viral RNA peaked by 3 dpi in pigs inoculated with rNJ0612NME6 and viral RNA was still detectable at 21 dpi, viral RNA peaked at 5 dpi in M51R inoculated animals and fell below level of detection by 14 dpi. These differences were more profound in contact-infected groups (**Figure 5A**). Similarly, infectious virus was recovered at higher titers and for longer periods in rNJ0612NME6 directly inoculated and contact animals. In M51R-infected animals, virus was recovered at lower titers and only from directly inoculated animals (**Figure 5B**). Virus was not recovered in nasal swabs of M51R contact exposed pigs. The peak of virus recovery in both groups was consistent with the rupture of the vesicles in the snout (**Figure 5B**).

No significant differences in oral shedding of viral RNA were observed between groups of pigs infected with either of the two viruses. Viral RNA was detectable as early as 1 dpi and 1 dpe in directly inoculated or contact exposed pigs, respectively. Oral shedding peaked by 4 dpe and 5 dpi and decreased steadily for both viruses until day 14. By 21 dpi (20 dpe), only animals inoculated with rNJ0612NME6 had detectable viral RNA in oral swabs (**Figure 6A**). Infectious virus was detected intermittently and in low titers in oral swabs collected from pigs infected with either virus. Higher average titers of infectious virus were recovered from pigs

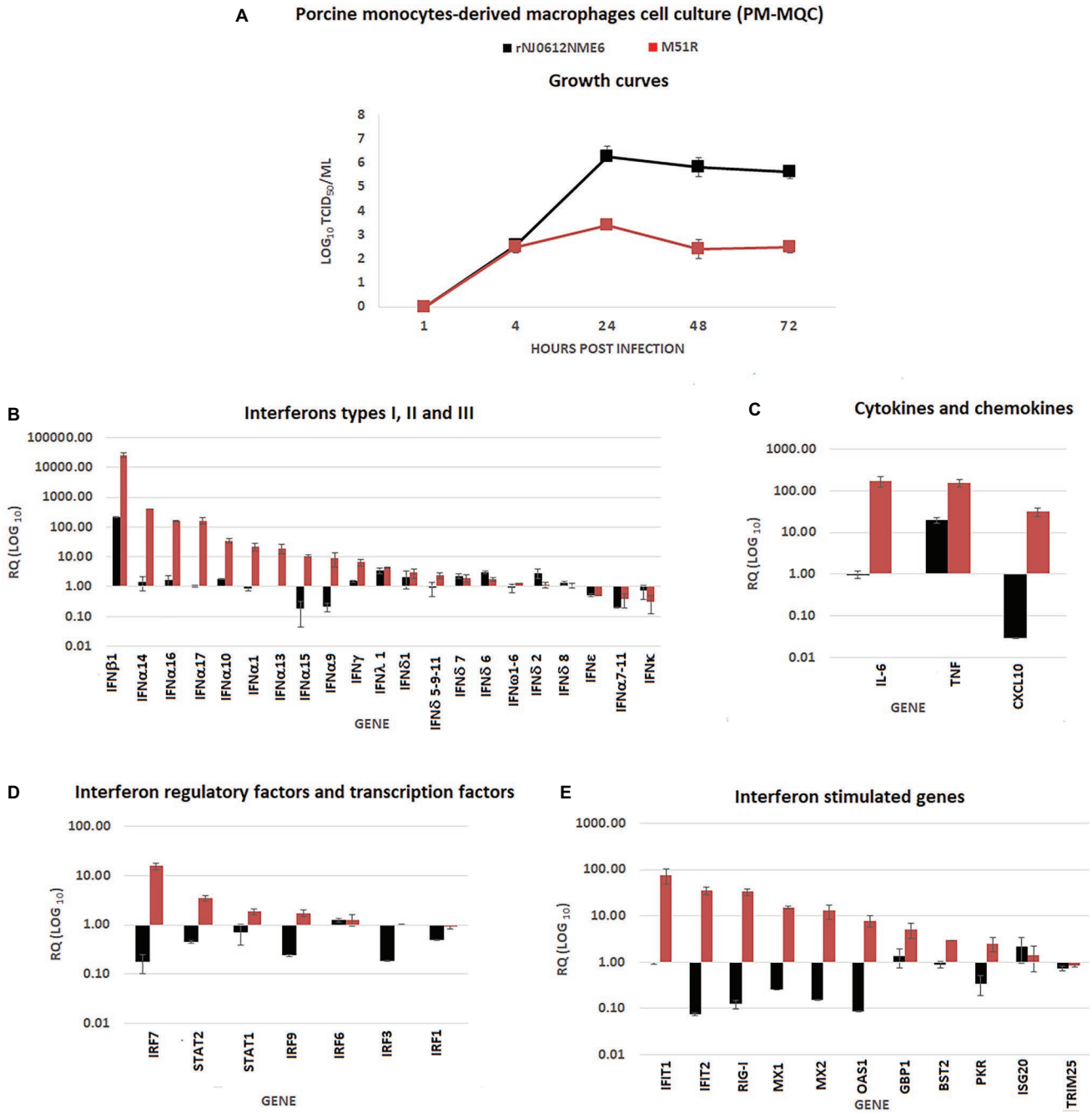

FIGURE 2 | *In vitro* characterization of rNJ0612NME6 and M51R viruses in porcine monocyte-derived macrophages cell cultures (PM-MQC). **(A)** Multistep growth curves were performed in PM-MQC to compare virus yields between both viruses at specific times post-infection, using at initial MOI = 0.01. Differences in the transcriptional immune gene response induced by different viruses on PM-MQC, were assessed by qRT-PCR. RQ values represent relative quantities of mRNA accumulation (estimated by $2^{-\Delta\Delta Ct}$) with their corresponding SD in **(B)** type I, II and III IFN, **(C)** cytokines and chemokines, **(D)** Interferon regulatory factors, and transcription factors, and **(E)** Interferon stimulated genes. All experiments were conducted in triplicates.

infected with rNJ0612NME6 than with M51R between 2 and 6 dpi regardless the route of infection (**Figure 6B**).

RNAemia

Although infectious VSV is not detectable in blood, the presence of RNAemia in experimentally infected pigs was first reported in a previous study as a potential marker for VSV virulence (Velazquez-Salinas et al., 2018a). The ability of rNJ0612NME6 and M51R to induce RNAemia (presence of viral RNA in blood) was assessed by rRT-PCR in whole blood samples collected during the course of the experiment.

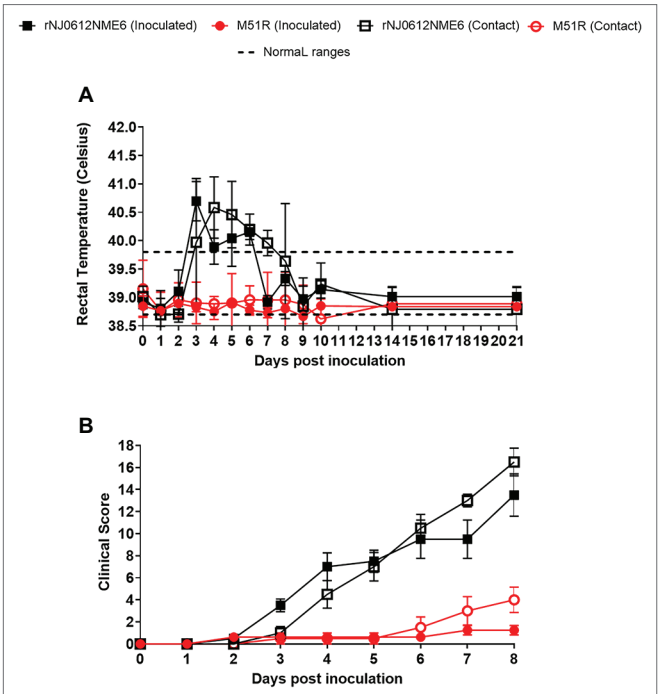

FIGURE 3 | Clinical evaluation in pigs. Two independent groups of pigs were inoculated with 1×10^7 $TCID_{50}$ with either rNJ0612NME6 or M51R viruses. **(A)** Changes in rectal temperatures, denoting fever in pigs direct inoculated or infected by contact exposure within each group, were monitored daily during the experiments. Dashed lines represent the standard rectal temperature of an uninfected pig. **(B)** The development of vesicular lesions in snout, mouth, lips, feet was examined daily in both groups of pigs. In both groups, maximum clinical scores were reached at 8 dpi. In both cases values represent means and standard deviations of cumulative clinical scores within each group of pigs.

Consistent with its inability to induce severe clinical signs in pigs, animals infected with M51R did not have detectable RNAemia regardless of the route of infection (**Figure 7**). In contrast, regardless of the route of infection, rNJ0612NME6 infection resulted in severe clinical signs and a marked phase of RNAemia during the acute stage of infection. Levels of viral RNA in the blood of these pigs peaked at 4 dpi or 3 dpe and dropped below levels of detection by 7 dpi or dpe (**Figure 7**).

Consistent with previous findings (Velazquez-Salinas et al., 2018a), despite high levels of RNAemia in pigs infected with rNJ0612NME6, attempts to isolate infectious virus were unsuccessful despite using different cell lines (BHK-21 and Vero cells) and dilution of blood samples to mitigate the action of potential inhibiting factors.

Systemic Type I IFN Response

To evaluate induction of systemic type I IFN response in groups of pigs after infection with the different viruses, serum samples collected during the acute phase were evaluated by Mx-CAT-ELISA. In contrast to results in primary cells, where M51R was able to induce higher amounts of type I IFN than the parental virus, pigs infected with rNJ0612NME6 developed a faster and higher systemic type I IFN response than pigs infected with M51R (**Figure 8**).

Comparison between groups of pigs infected by the same route with different viruses showed statistically significant ($p < 0.05$) differences in IFN levels at 3 dpi. In M51R contact exposed pigs, IFN response was delayed, reflecting the delay in the development of vesicular lesions observed in this group of pigs.

Adaptive Immune Response

To evaluate the adaptive immune response, neutralizing antibodies were quantified by serum neutralization assay. Regardless of the virus, directly inoculated pigs developed similar antibody responses starting at 6 dpi. However, by 14 and 21 dpi, pigs inoculated with rNJ0612NME6 had significantly higher antibody levels ($p < 0.05$) than those inoculated with M51R (**Figure 9**).

Neutralizing antibody response in pigs infected by contact exposure with rNJ0612NME6 was detectable at 6 dpe, while this response was detectable around 9 dpe in pigs infected with M51R. By the end of the experiment, the level of neutralizing antibodies was ~100 fold higher in pigs infected by contact with rNJ0612NME6 than in those infected with M51R (**Figure 9**).

Virus Detection in Tissues

Post mortem tissue collection was done at 21 dpi or dpe from the two pigs from each group with the highest clinical scores. Remarkably, despite their contrasting difference in virulence, no statistically significant difference was seen in the amount of viral RNA detected in the different tissues from rNJ0612NME6 and M51R infected pigs (**Figure 10**). Increased amounts of viral RNA was found in lymphoid tissues associated with anatomic sites of local replication including the tonsil of the soft palate, submandibular lymph node, and popliteal lymph node. Although not statistically significant, higher level of viral RNA was observed in the spleen collected from pigs infected with rNJ0612NME6 than in those infected by M51R, consistent with the increased capacity of rNJ0612NME6 virus to systemically disseminate during infection in pigs. Interestingly, despite the absence of RNAemia in the group of pigs infected with M51R virus, similar levels of viral RNA were found in skeletal muscle collected from pigs in both groups, suggesting that muscle tissue might play a role during the VSV infection in pigs (**Figure 10**). Finally, no infectious virus was recovered from any of the post mortem tissues collected during this study.

DISCUSSION

In general, very little is known about virus-host interactions during the infection of VSNJV in natural hosts. Understanding these processes is key to assessing intrinsic viral factors determining virulence in domestic animals. In a previous study, we reported that the increased virulence of the epidemic VSNJV strain NJ0612NME6, compared with its genetically closest endemic

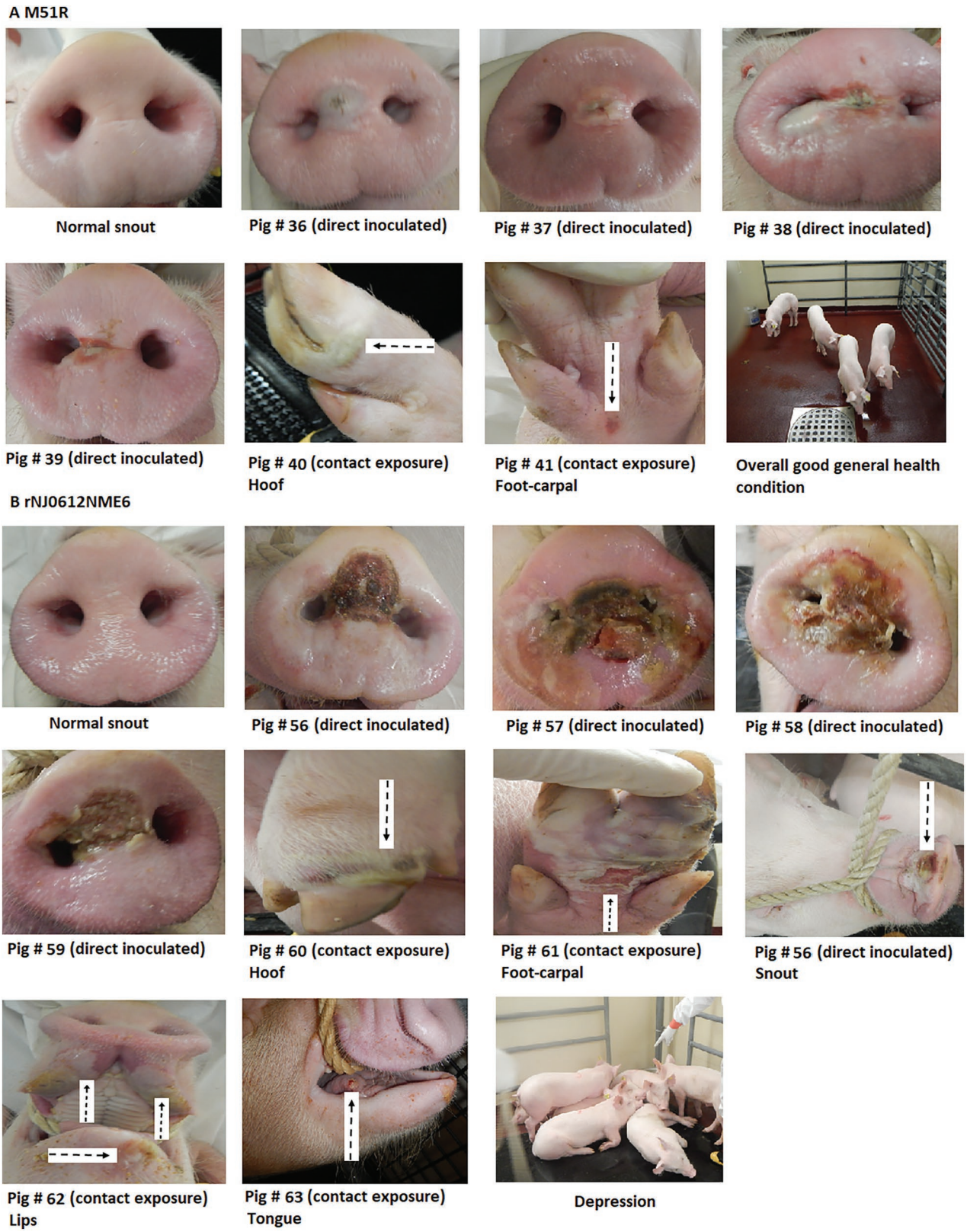

FIGURE 4 | Clinical differences between groups of pigs infected with either rNJ0612NME6 or M51R viruses. **(A)** Despite the intrinsic characteristics of M51R virus to overcome the host innate immune response, clinical outcomes from different pigs infected within this group were characterized by the development of vesicular

(Continued)

FIGURE 4 | lesions in both direct inoculated and contact exposure pigs. Overall pigs in this group kept a general health condition during the entire experiment (absence of fever, general depression, and lameness). **(B)** Contrasting differences were observed in pigs infected with rNJ0612NME6 in terms of vesicular lesion size development, systemic dissemination characterized for the development of vesicular lesions at different anatomic sites, and overall alteration of the health condition, especially during the acute phase of the infection.

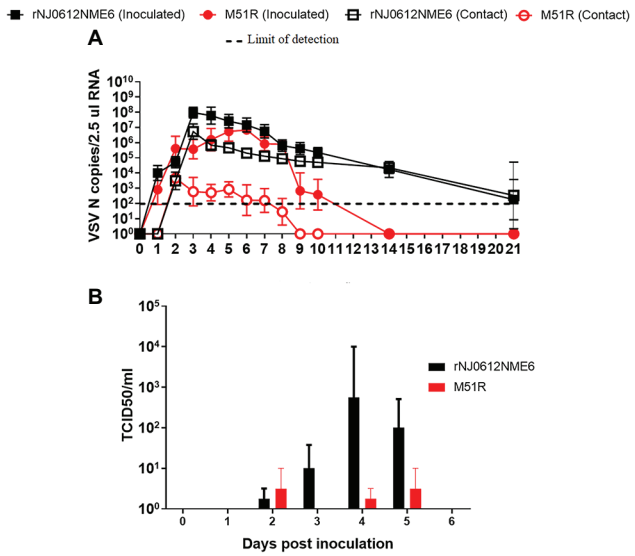

FIGURE 5 | Differences of viral shedding on nasal swabs between groups of pigs infected with either rNJ0612NME6 or M51R viruses. **(A)** Levels of Vesicular stomatitis virus (VSV) nucleocapsid (N) RNA in different samples were quantified by rRT-PCR. Limit of detection of qRT-PCR using for this study was previously determined to be about 1.98 VSV N copies/2.5 µl RNA. **(B)** Viral isolations were conducted on Vero cells, and then viral titers from positive samples were determined and expressed in $TCID_{50}$/ml. Values represent means and standard deviations within different groups of pigs.

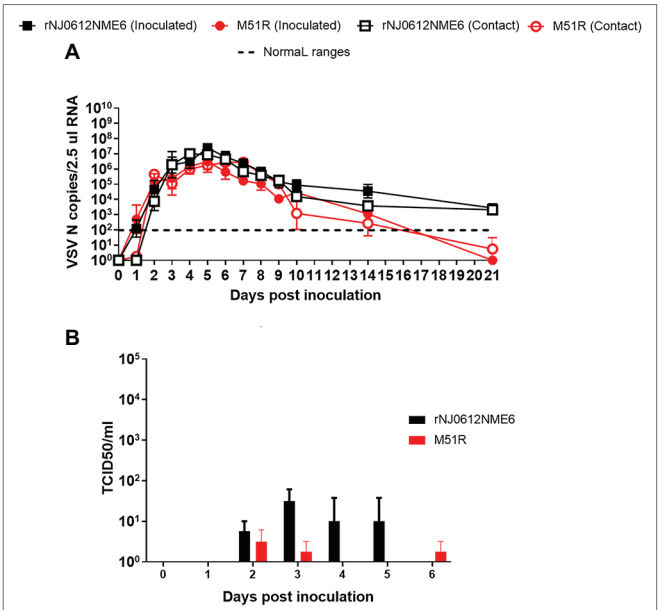

FIGURE 6 | Differences of viral shedding on oral swabs between groups of pigs infected with either rNJ0612NME6 or M51R viruses. **(A)** Levels of VSV nucleocapsid (N) RNA in different samples were quantified by rRT-PCR. Limit of detection of qRT-PCR using for this study was previously determined to be about 1.98 VSV N copies/2.5 µl RNA. **(B)** Viral isolations were conducted on Vero cells, and then viral titers from positive samples were determined and expressed in $TCID_{50}$/ml. Values represent means and standard deviations within different groups of pigs.

relative, might be associated with the ability of the former virus strain to modulate the innate immune responses in pigs (Velazquez-Salinas et al., 2018a). In this study, we introduced into NJ0612NME6 the M51R mutation shown to affect the viral ability to overcome innate responses (Ahmed et al., 2003; Rajani et al., 2012, Hastie et al., 2013; Melzer et al., 2017), to gain more insights about the role of innate responses during VSV infection in pigs. The outcomes of this study demonstrated that there is a positive correlation between the ability of VSNJV to replicate and overcome the innate immune responses in cultured macrophages and virulence in pigs.

In vitro studies were carried out in two types of primary cells: FPKC (non-immune cells) and PM-MQC (immune cells), representing different cellular environments that might be encountered by the virus during infection in pigs. Surprisingly, no significant differences in growth kinetics, viral yield, and plaque formation were seen between rNJ0612NME6 and M51R in FPKC despite significant differential transcription of ISG genes and the production of increased amounts of type I IFN observed in M51R-infected FPKC.

On the other hand, the M51R mutant had impaired growth kinetics in PM-MQC, indicating the essential role played by this residue in the replication of VSNJV in macrophages. The difference between FPKC and PM-MQC supporting replication of M51R may be explained by the intrinsic immune characteristics of each cell type and suggests that immune cells may play a critical role in VSNJV pathogenesis in pigs. While fibroblasts and epithelial cells are predominately producers of IFNβ (Ivashkiv and Donlin, 2014), macrophages express different pattern recognition receptors, and the constitutive expression of ISG like IRF-7 (a regulator of multiple of type I IFN), that allow these cells to mount a rapid antiviral response, reacting to initial picomolar concentrations of type I IFNs (Ning et al., 2011; Lazear et al., 2013; Ivashkiv and Donlin, 2014). This is consistent with the differences in the transcriptional profiles found between PM-MQC and FPKC after infection with rNJ0612NME6 and M51R, where the transcriptional profile of IRF-7 and IFN α in FPKC was not different between the two viruses.

Several studies have shown the relevant role that type I IFNs play as antiviral cytokines against VSV infection (Muller et al., 1994; Stojdl et al., 2003; Lichty et al., 2004). However, our results suggest that the effectiveness of type I IFNs against VSNJV in epithelial cells might vary depending

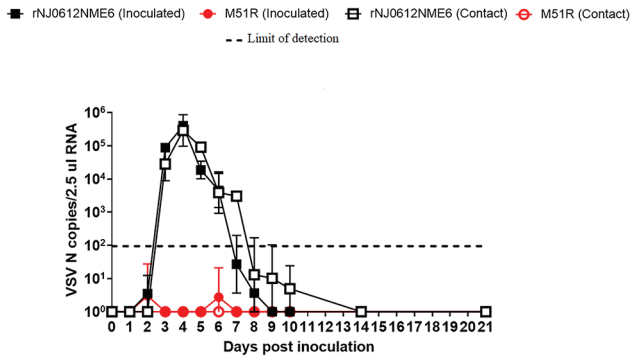

FIGURE 7 | Differences of RNAemia levels between groups of pigs infected with either rNJ0612NME6 or M51R viruses. Levels of RNAemia were quantified by rRT-PCR from blood samples collected daily from all pigs during the course of this experiment. Limit of detection of qRT-PCR using for this study was previously determined to be about 1.98 VSV N copies/2.5 µl RNA. Values represent means and standard deviations within different groups of pigs.

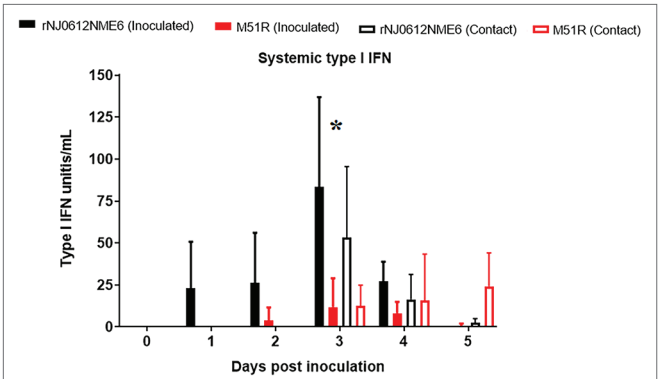

FIGURE 8 | Differences in systemic levels of type I IFN induction between groups of pigs infected with either rNJ0612NME6 or M51R viruses. Mx-CAT reporter assay was used to monitor the systemic type I IFN production in pigs during the acute phase of the infection with different viruses. Values represent means and standard deviations within different groups of pigs. Asterisk at 3 dpi shows significant differences ($p < 0.05$) between pigs infected with rNJ0612NME6 and M51R viruses.

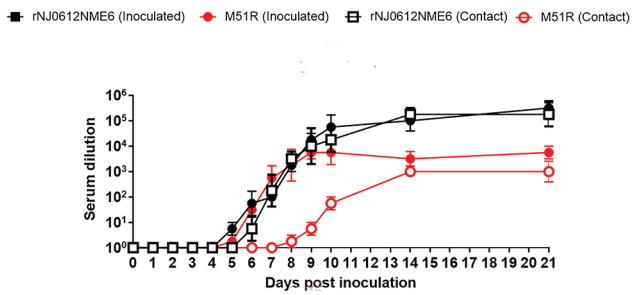

FIGURE 9 | Differences in neutralizing antibody production between groups of pigs infected with either rNJ0612NME6 or M51R viruses. Viral neutralization assay was used to monitor the development of neutralizing antibody titers in pigs, using serum samples collected during the course of this experiment. Values represent means and standard deviations within different groups of pigs.

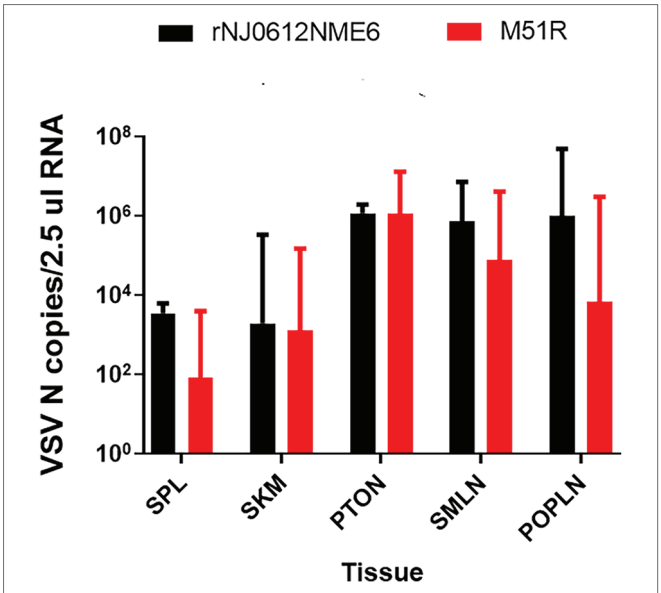

FIGURE 10 | Differences in viral RNA accumulation in postmortem tissues between groups of pigs infected with either rNJ0612NME6 or M51R viruses. Postmortem tissues were collected at 21 dpi, and analyzed by qRT-PCR to detect VSV nucleocapsid RNA. Limit of detection of qRT-PCR using for this study was previously determined to be about 1.98 VSV N copies/2.5 µl RNA. Values represent means and standard deviations within different groups of pigs. Abbreviations: tonsil of the soft palate (PTON), submandibular lymph node (SMLN), spleen (SPL), snout skin (SNT), popliteal lymph node (PopLN), and skeletal muscle (SKM).

on the source of IFN, whether it is external or internally induced. On one hand, our results showed the competence of FPKC to mount an effective antiviral state against the infection of either rNJ0612NME6 or M51R by prior stimulation with an external source of type I IFN. This is consistent with a previous report, showing that the oncolytic virus VSV-IFNβ-NIS coding for the human IFNβ (Naik and Russell, 2009) had an attenuated phenotype in FPKC and *in-vivo*, failing to produce epithelial lesions on the skin of infected pigs (Velazquez-Salinas et al., 2017).

On the other hand, we showed the inability of virus-induced type I IFN to fully contain an infection with M51R virus not only in FPKC, but remarkably also *in vivo*, as demonstrated by the formation of epithelial lesions (albeit small) in the snout of pigs direct inoculated with this virus. This suggests that epithelial cells of the skin might be less competent to mount an adequate antiviral response against VSNJV under natural infection. On this perspective, it has been reported that during natural infections in livestock, local epithelial lesions (without generalization) occur even in animals with significant levels of neutralizing antibodies induced by prior infection, suggesting that local processes might mediate lesion development (Rodriguez et al., 1990).

We have now shown that the M51R substitution in the matrix protein of VSNJV has a profound impact on the *in vivo* virulence of this virus, compromising its ability to systemically disseminate in infected pigs and thus reducing the formation and size of secondary vesicular lesions and the presentation of clinical signs including fever and obtundation. Interestingly,

the correlation between the reduction in the capacity of M51R virus to replicate *in vitro* in macrophage cultures and the impairment to systemically disseminate *in vivo* is consistent with previous studies that show the essential role macrophages play to clear VSV during infection in mice by preventing systemic dissemination, supporting cytokine production, and facilitating the initiation of immune humoral responses (Ciavarra et al., 2005, 2006; Junt et al., 2007).

Furthermore, the clinical outcome in pigs infected with M51R resembles that shown by pigs experimentally infected with an attenuated VSIV strain, where clinical infection is characterized by the formation of vesicular lesions at the site of inoculation but the formation of secondary vesicular lesions and the presentation of fever are rarely observed (Martinez et al., 2003; Stallknecht et al., 2004). This suggests that there might be important differences between different strains in their ability to overcome the innate immune response in pigs. It has been suggested that interferon induction (or suppression) is a *quasi-species* marker in VSV. This is supported by prior observations that VSNJV field isolates derived from individual local lesions vary in their ability to repress induction of type I IFN (Marcus et al., 1998).

In addition, we detected the presence of viral RNA in pig tissues collected from both groups at 21 dpi, but we were not able to isolate infectious virus from any of those samples. This is consistent with previous studies in mice (Simon et al., 2010), cattle (Letchworth et al., 1996), and hamsters (Barrera and Letchworth, 1996), where the presence of viral RNA but no viral mRNA was detected. No infectious virus was detected at this stage of the disease indicating that VSV genomic or subgenomic RNA can persist in tissues from convalescent animals for long periods of up to 5 months (Letchworth et al., 1996). Future studies should be conducted to understand the acute infection dynamics and mechanisms of early VSV infection in pigs leading to the persistence of viral RNA (Stenfeldt et al., 2014). The M51R mutant virus could be a useful tool in studies to understand the role of different tissues and immune responses in VSV infection of pigs.

Finally, our results showed that the levels of systemic type I IFN were not associated with clinical signs in pigs infected with rNJ0612NME6 or M51R. These results contrast previous results comparing epidemic and endemic strains of VSNJV (Velazquez-Salinas et al., 2018a), where decreased type I IFN was seen in animals with severe disease. Our results showed that increased levels of type I IFN induced during infection are not always associated with protection in pigs and in some cases might act as a marker of disease at least at the local epithelium level. More studies are necessary to evaluate the role of innate immune responses mounted by different immune cell types involved in immune responses to infection with VSNJV.

Importantly, the contrasting clinical differences between pigs infected with rNJ0612NME6 or M51R confirm the relevance of RNAemia, fever, and intensity of antibody immune response as appropriate biological markers of virulence during the characterization of VSV strains in pigs. In the case of RNAemia, disparate clinical scores between groups of pigs infected with different viruses suggest that epithelial lesions are associated with RNAemia.

Also, despite its inability to fully inhibit innate responses, M51R was still capable of producing vesicular local lesions at inoculated sites, suggesting that other factors are relevant during local replication of VSNJV, whereas attenuated VSIV cause reduced or no lesions at the inoculation site (Martinez et al., 2003, 2004).

CONCLUSION

Collectively, our results show that a highly virulent VSNJV containing a single amino acid mutation (M51R) is significantly attenuated in pigs and has impaired growth in pig immune cells, which is likely associated to increase type I IFN responses. The mutant virus retains its ability to grow in epithelial cells and can replicate locally in inoculated sites in pigs, suggesting different mechanisms of pathogenesis for local and systemic VSNJV infections. Furthermore, our results show the relevance of the M51R mutant as a model to better understand the immune interactions between VSNJV and other relevant natural hosts such as cattle and horses (Rodriguez, 2002). Based on previous studies, different clinical outcomes might be expected between pigs and cattle and horses during the infection with M51R (Howerth et al., 2006; Scherer et al., 2007; Mead et al., 2009).

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the PIADC Institutional Animal Care and Use Committee of the US Departments of Agriculture and Homeland Security (protocol number #245-05-14R).

AUTHOR CONTRIBUTIONS

LV-S, SP, AV-R, and LR conceived and designed the experiments. LV-S, SP, CS, LH, DG, SR, and EB performed the experiments. LV-S, SP, and LR analyzed the data. LR, MB, and JA contributed the reagents, materials, and analysis tools. LV-S, SP, CS, MB, LH, DG, EB, SR, AV-R, JA, and LR wrote the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CAPÍTULO 10

Discusión

Los resultados de esta investigación mostraron por primera vez las diferencias biológicas entre una cepa epidémica del VSNJV y su pariente endémico más cercano, mostrando que la cepa epidémica NJ0612NME6 representa un fenotipo más virulento para el cerdo, en comparación con la cepa endémica NJ0806VCB [70]. De manera consistente con la hipótesis planteada en esta investigación, el incremento en la virulencia mostrado por la cepa NJ0612NME6 fue clínicamente demostrado con base al aumento en el número de lesiones vesiculares producidas en los cerdos infectados con esta cepa, en comparación con el grupo de cerdos infectados con la cepa NJ0806VCB. En este sentido, las diferencias en la regulación de diferentes citoquinas evaluadas durante la infección en ambos grupos de cerdos, las cuales incluyeron: la interleucina seis (IL-6), el factor de necrosis tumoral (TNF) y el interferón tipo I (IFN1), sugieren que, la cepa NJ0612NME6, podría tener una mayor capacidad para regular la respuesta inmune innata del huésped vertebrado. Estos hallazgos son discutidos con detalle a continuación.

Diferencias genómicas entre las cepas NJ0612NME6 (1.1) y NJ0806VCB (1.2).

Con la finalidad de descifrar el número y la localización de diferencias nucleotídicas distintivas entre la cepa epidémica NJ0612NME6, y su pariente endémico más cercano la cepa NJ0806VCB, se obtuvieron las secuencias genómicas completas de los virus NJ0612NME6 y NJ0806VCB, las cuales fueron publicadas en la base de datos del *GenBank* (Capitulo 5). Desde nuestra perspectiva, la información genética contenida en estas secuencias será de mucha utilidad para conducir futuros trabajos de investigación enfocados en entender las fuerzas evolutivas asociadas a la evolución de linajes epidémicos del VSNJV.

Actualmente, las diferencias nucleotídicas entre cepas epidémicas y endémicas del VSNJV son poco conocidas. Típicamente, los estudios evolutivos implicando brotes epidémicos del VSNJV en los Estados Unidos, se han llevado a cabo utilizando secuencias parciales del gen P (región hipervariable), por lo que la información obtenida a partir de esta metodología ha sido limitada [1, 12, 4]. En este sentido, los resultados presentados en esta tesis doctoral

muestran por primera vez las diferencias nucleotídicas distintivas a lo largo del genoma, entre una cepa epidémica del VSNJV y su pariente endémico más cercano, hecho que adquiere más relevancia, considerando el incremento en la virulencia mostrado por la cepa epidémica NJ0612NME6 durante la caracterización *in vivo* llevada a cabo en el cerdo, en comparación con lo observado con la cepa endémica NJ0806VCB (Capitulo 6) [70] .

Los resultados, mostraron un patrón complejo de substituciones distintivas entre ambas cepas, el cual está dominado primordialmente por la acumulación de mutaciones sinónimas a lo largo del genoma de estos virus, que en primera instancia podría ser explicado por efectos de la selección negativa, con la finalidad de preservar la funcionalidad del proteoma viral [71].

Sin embargo, desde otra perspectiva, nuestros resultados fueron consistentes con el patrón evolutivo de mutaciones sinónimas mostrado por el VSIDV durante su propagación *in vitro* bajo condiciones de selección positiva [72], por lo que estas similitudes nos permitirían proponer la hipótesis alterna de que, la selección positiva de mutaciones sinónimas durante la evolución natural del VSNJV, podría estar favoreciendo las características fenotípicas de los linajes epidémicos del VSNJV. Esta hipótesis es por demás interesante, si consideramos que, aunque la función exacta de las mutaciones sinónimas no es del todo conocida, la introducción experimental de mutaciones sinónimas en el gen L del VSIDV, produjo un detrimento en la virulencia de este virus, al ser evaluado en un modelo de infección en ratón [73]. Otro ejemplo que sustenta esta hipótesis fue el mostrado en el virus de fiebre porcina clásica, donde, la introducción experimental de múltiples mutaciones sinónimas a lo largo de la región codificante de la proteína E2, atenuó *in vivo* a la cepa Brescia, una cepa altamente virulenta que produce una mortalidad elevada en porcinos [74].

Adicionalmente, otra posible explicación al patrón evolutivo de mutaciones sinónimas reportado entre estas dos cepas virales podría ser debido a la adaptación a los diferentes vectores utilizados por el virus NJ0612NME6 en la naturaleza, como resultado de su necesidad a adaptase a diferentes ambientes ecológicos durante la epizootia. Esta hipótesis podría sustentarse en un estudio llevado a cabo en Drosophila, donde se mostró la

importancia que el RNA de interferencia (iRNA) tiene en la respuesta inmunológica de este insecto contra la infección por el VSV, controlando la replicación viral [75].

En este sentido, la adaptación del VSNJV a los insectos podría significar la acumulación de diferentes mutaciones sinónimas y no sinónimas a lo largo del genoma, en respuesta a la acción del iRNA. Es Interesante mencionar que, la acción conjunta del iRNA, junto con algunas retrotranscriptasas en el insecto, parecen jugar un papel esencial en el establecimiento de ciclos de persistencia, los cuales son vitales para el mantenimiento del virus en el insecto [76]. Esto podría ser especialmente relevante, considerando el papel que los insectos podrían estar jugando durante el mantenimiento y la expansión del VSNJV en los eventos epizoóticos [3].

El patrón de mutaciones no sinónimas reportado en esta investigación fue consistente con lo encontrado en una comparación similar llevada a cabo en el virus de encefalitis equina venezolana, donde se muestra que, el incremento de la virulencia mostrado por las cepas epidémicas de este virus, en comparación con sus ancestros endémicos, podría estar asociada a la acción conjunta de diferentes substituciones no sinónimas a lo largo del genoma de este virus [5]. Actualmente en el caso del VSV, poco es conocido sobre el impacto en la virulencia producido por las diferentes proteínas virales.

Como se detalla en la introducción de esta tesis, la proteína M es una de las proteínas más estudiadas en el VSV, la cual se encuentra implicada directamente en los mecanismos de virulencia de este virus, controlando la respuesta inmunológica del hospedador vertebrado durante la infección [19, 20.] Sin embargo, y a pesar de las diferencias en los niveles de virulencia mostrados entre las cepas NJ0612NME6 y NJ0806VCB, no se encontró ninguna diferencia no sinónima en el gen M durante la comparación genómica realizada entre ambas cepas, indicando que otras proteínas más allá de la proteína M, están regulando la virulencia del VSNJV en el cerdo. En este sentido, los resultados de esta tesis muestran un patrón discreto de substituciones no-sinónimas entre ambos virus, el cual incluye principalmente a los genes P, G y L. lo cual podría soportar la hipótesis de que la acción de diferentes proteínas

está regulando la virulencia del VSNJV en el cerdo. Un fenómeno que sería consistente con lo reportado en otros arbovirus como el virus de encefalitis equina venezolana [15, 18].

De manera interesante, constituyendo tan solo el 7.7% del total del total de las regiones codificantes en el genoma del VSNJV, en el gen P se encontraron casi la mitad de las mutaciones no sinónimas distintivas entre ambas cepas virales. En este contexto, una investigación reciente utilizando el VSIDV indica el papel que juega la proteína P en la resistencia contra la acción antiviral producida por la proteína *Tripartite Motif Containing 69* (TRIM69) [77], haciendo esta proteína un blanco interesante para la realización de futuras investigaciones para entender la patogenia del VSNJV en los animales domésticos. Asimismo, indirectamente los resultados de una investigación llevada a cabo en el virus primaveral de la carpa, otro *vesiculovirus* de la familia *Rhabdoviridae*, muestran que la función de a la proteína P se encontró ligada a la capacidad de este virus para regular la producción del interferón tipo 1 (IFN1), mediante la interacción de esta proteína P con la fosfoquinasa TBK1, produciendo así, una disminución en la fosforilación del factor de regulación del interferón 3 (IRF3), el cual es esencial para estimular la producción de IFN1 en las células infectadas [78].

Otro hallazgo interesante encontrado durante el análisis de sustituciones no-sinónimas en el gen P, fueron las diferentes sustituciones que se encontraron impactando las proteínas C y C', codificadas dentro de un segundo marco de lectura en este gen [25]. Aunque en el caso del VSV, la función de estas proteínas es desconocida, su elevada homología entre los diferentes aislamientos del VSV en el campo, supone que podrían jugar un papel en la virulencia de este virus [25]. Esta aseveración podría ser sustentada en base a un estudio llevado a cabo en paramixovirus, donde la función de la proteína C aparece relacionada con el control a la repuesta antiviral, mediante la inhibición de la fosforilación del factor de regulación del interferón siete (IRF7) [79]. Este resultado es por demás interesante, ya que podría ayudar a comprender nuestros resultados *in vitro*, con relación a la mayor habilidad mostrada por el virus NJ0612NME6 para controlar la transcripción de IRF7 durante la infección en células primarias de macrófagos porcinos.

En el caso del gen G, la comparación reveló múltiples substituciones no sinónimas entre ambos virus. En este sentido, un estudio comparativo previo entre las glicoproteínas de los virus VSNJV y VSIDV mostró el papel que esta proteína juega en la virulencia en el cerdo, sin que el mecanismo especifico haya sido determinado [63]. Este hallazgo, podría ayudar a soportar los resultados obtenidos en esta tesis doctoral, señalando al gen G como un blanco interesante para llevar a cabo futuros estudios de genética reversa dirigida. De manera interesante, las mutaciones encontradas en el presente trabajo entre los residuos 1068 y 1361 de la proteína G, se encuentran dentro de una zona presumiblemente implicada en el control de la inducción del IFN1 del VSV, la cual fue previamente evidenciada durante experimentos *in vitro* [38, 80]. Estos resultados, ofrecen otra alternativa para explicar las diferencias en la habilidad para controlar la inducción de interferón sistémico *in vivo* entre las cepas NJ0612NME6 y NJ0806VCB. Adicionalmente, es posible sugerir que las diferencias observadas entre ambos virus en esta proteína, podrían haber aumentado la capacidad viral de la cepa NJ0612NME6 para evadir la acción de algunas proteínas con acción antiviral, tal y como lo menciona un estudio reciente, donde se muestra el papel de la proteína G del VSIDV como un antagonista de la proteína BST2 (*Bone Marrow Stromal Cell Antigen 2*), una proteína antiviral inducida por la acción del IFN1, la cual tiene como mecanismo de acción sobre VSIDV, el afectar la salida de partículas virales infecciosas de las células infectadas [81].

Finalmente, un número importante de sustituciones no-sinónimas (~39%) se localizaron en el gen L entre estos dos virus. Al igual que las demás proteínas en el VSV, se conoce muy poco sobre el papel que juega la polimerasa como promotora de la virulencia de este virus. Al respecto, se ha demostrado que mutaciones que alteran la región encargada de controlar la metilación de la caperuza en el ARN mensajero del virus (CRVI), tienen un efecto negativo en la virulencia del VSIDV en ratones [82]. Curiosamente, el patrón de mutaciones detectado en nuestra investigación no impacta la región CR-VI, pero si, la región CR-V asociada con la formación de la caperuza en el ARN mensajero (residuo 2513), lo que podría suponer que un aumento en la estabilidad de los mensajeros del virus NJ0612NME6 podría representar otro factor de virulencia en este virus.

Sin embargo, al igual que los residuos 398, 549,1526, 2506, 2941 y 3362 situados en diferentes proteínas del virus, el residuo 2513 no aparece conservado entre los otros virus evaluados pertenecientes al linaje 1.1. De manera interesante, todos estos residuos aparecen conservados entre los virus NJ0806VCB (linaje 1.2) y NJ1008JAB, un virus perteneciente al linaje 1.1 pero colectado en el estado de Jalisco, México durante el año 2008, sugiriendo que estos cambios podrían reflejar más un aspecto ligado a la adaptación ecológica del virus en insectos, más que a la capacidad del linaje 1.1 para aumentar la virulencia en el hospedador vertebrado.

Subsecuentes análisis de evolución utilizando una población viral estadísticamente significativa de virus pertenecientes al linaje 1.1, son necesarios para determinar tanto los mecanismos evolutivos como los sitios relevantes que promovieron la evolución de este linaje epizoótico, permitiendo así, inferir con mayor certeza la relevancia de los diversos residuos en el proteoma viral descritos en esta tesis doctoral.

Caracterización biológica en el cerdo.

Evaluación clínica.

El estudio comparativo de patogenia llevado a cabo en el cerdo entre los virus NJ0612NME6 y NJ0806VCB, permitió inferir diferencias biológicas entre el linaje epidémico 1.1 representado por la cepa NJ0612NME6, y su pariente endémico más cercano el linaje 1.2 representado por la cepa NJ0806VCB. Los resultados de este estudio de patogenia soportaron nuestra hipótesis de investigación, indicando que la cepa NJ0612NME6 representa un fenotipo más virulento para el cerdo, que la cepa NJ0806VCB.

Los resultados del estudio de patogenia llevados a cabo en esta investigación, fueron consistentes con los resultados de estudios previos en cerdos, donde se muestra la utilidad de esta especie domestica como modelo experimental para valorar la virulencia en el VSV [6, 61, 62, 63, 65, 66, 83, 84.] Clínicamente, una de las características biológicas distintivas entre las cepas NJ0612NME6 y NJ0806VCB determinadas durante esta investigación, fue la mayor capacidad de virus epidémico para inducir un número mayor de lesiones epiteliales en los

cerdos infectados por este virus. Este resultado es por demás interesante, ya que, en ausencia de una fase de viremia durante la infección con el VSV en animales domésticos, la presencia de lesiones vesiculares ha sido consideradas como la principal fuente de virus para los vectores [69], o para producir contagio por directo a otros animales domésticos [62].

En este sentido, sería razonable suponer que la presencia de un fenotipo virulento como el virus NJ0612NME6, con una capacidad aumentada para inducir un mayor número de lesiones vesiculares en los animales infectados, podría representar una característica determínate para la presentación de una epizootia. No obstante, es importante considerar que, la emergencia en los arbovirus es un fenómeno complejo, el cual tiene que ser comprendido desde un enfoque multifactorial [56], por lo que, el aumento de virulencia de mostrado en la cepa NJ0612NME6 podría representar solo uno de los eslabones en la cadena de eventos.

Otra diferencia clínica entre los grupos de cerdos infectados con las diferentes cepas usadas en este estudio fue la capacidad de la cepa NJ0612NME6 para inducir niveles más elevados de fiebre en los animales infectados con este virus. Aunque este signo clínico ha sido típicamente asociado con la virulencia en otras infecciones virales como fiebre porcina clásica [74], peste porcina clásica [85] y fiebre aftosa [86], en el caso del VSV la relación entre esta condición fisiológica y virulencia en el cerdo, no ha sido considerada, debido a que este signo clínico ha sido descrito como raro o poco frecuente durante la infección experimental en cerdos [61, 62, 63 87, 88].

Sin embargo, contrastando con lo antes publicado, los resultados de esta investigación doctoral mostraron una correlación entre la intensidad de la fase febril y la virulencia de las cepas usadas en este estudio. En este contexto, es importante considerar que los hallazgos descritos en esta tesis podrían diferir con lo antes publicado debido principalmente a la diferencia entre las cepas utilizadas en los diferentes estudios. En nuestro estudio, la estrecha relación genética entre ambas cepas podría explicar la capacidad de ambas cepas para inducir procesos febriles durante la fase aguda de la infección. Sin embargo, es razonable proponer que la caracterización de un mayor número de cepas del VSNJV es necesaria para obtener una conclusión más sólida sobre esta relación.

Por otro lado, es interesante mencionar que no obstante que la fiebre es una respuesta fisiológica a la infección, la cual es capaz de estimular las capacidades secretorias, fagocíticas y citolíticas de diferentes tipos celulares asociados con la respuesta inmune (neutrófilos, NK, macrófagos y células dendríticas) [89], evidencia experimental obtenida en el laboratorio a partir de trabajos *in vitro*, demostró que las temperaturas de crecimiento sobre 40º C, representan un factor selectivo para el VSIDV, produciendo que las variantes termoestables seleccionadas en este ambiente, muestren un incremento en su capacidad para evadir la neutralización de sueros policlonales provenientes de huéspedes naturales de este virus [90]. Aunque este resultado no explica las diferencias en virulencia descritas en nuestro estudio, si se pudiera correlacionar con la mayor termoestabilidad mostrada de la cepa NJ0612NME6 en nuestros estudios in vitro, ofreciendo una posible explicación a la incrementada virulencia mostrada por esta cepa *in vivo*, no obstante, la capacidad de esta cepa para inducir procesos febriles intensos.

La observación anterior, podría representar una nueva hipótesis para poder explicar como la mayor virulencia en los fenotipos epidémicos del VSNJV, podría favorecer la presentación de signos clínicos como la fiebre y esta representar un ambiente selectivo para el aumento de variables con capacidad de evadir la respuesta neutralizante de los anticuerpos, incrementando así la replicación viral y la capacidad de transmisión durante las epizootias.

Evaluación de la dinámica de infección, mediante el uso de la prueba de la reacción de cadena de la polimerasa de tipo cuantitativa (qPCR).

RNAemia

A diferencia de otros trabajos previos [6, 61,62,63 65, 66, 87], es posible mencionar que un acierto en la planeación metodológica de nuestra investigación fue incluir el uso de la qPCR, como herramienta para evaluar la dinámica de infección en los cerdos infectados con cada uno de los virus usados en esta tesis. En este sentido, el uso de la qPCR hizo posible evidenciar por primera vez en cerdos infectados experimentalmente con el VSNJV, una fase de circulación de ARN viral en la sangre (RNAemia), durante la etapa aguda de la infección.

Este hallazgo es por demás interesante, ya que, de manera indirecta, estaría evidenciando la presencia de partículas virales circulando en la sangre de estos animales, mostrando la existencia de una fase potencial de viremia durante la infección del cerdo con el VSNJV. Sin embargo, debido a nuestra incapacidad para recuperar partículas virales infecciosas a partir de las muestras de sangre y suero colectadas de los cerdos durante la fase de RNAemia, en este punto no tenemos una explicación convincente sobre la relevancia biológica de este fenómeno. Es importante mencionar que la RNAemia es un fenómeno que ha sido descrito previamente no solo en equinos infectados experimentalmente con el VSV [64], sino también en bovinos infectados de manera natural en México con una cepa del VSNJV perteneciente al linaje 1.1[4], sin que alguna partícula infecciosa haya podio ser aisladas de las muestras sanguíneas provenientes de estos animales. Este resultado es consistente con la baja estabilidad mostrada por el VSV en ratones, donde se encontró que en animales inoculados de manera intravenosa no es posible recuperar virus infeccioso más allá de 10 minutos posteriores a la infección [92].

Por otro lado, la relación positiva entre la mayor intensidad de la fase RNAemia con la respuesta febril y el incremento de lesiones vesiculares mostrada por los cerdos infectados con el virus NJ0612NME6, permitiría proponer que la presencia e intensidad de esta fase podría ser usada como un marcador de virulencia, durante la caracterización experimental de cepas de campo, o virus mutantes del VSV en un modelo porcino.

Con la finalidad de encontrar una posible explicación a la falta de aislamiento de partículas infecciosas, en nuestra investigación demostramos la actividad antiviral intrínseca presente en el suero no inmune de cerdo contra el VSNJV, sugiriendo que la fase de RNAemia encontrada en la sangre de los cerdos infectados con ambos virus usados en nuestra investigación, podría representar una fase de viremia enmascarada por la acción de proteínas termolábiles (complemento) y termoestables presentes naturalmente en el suero no inmune de cerdo. Estos resultados fueron consistentes a lo observado en el suero de otras especies incluyendo humanos, ratones y perros [91, 92, 93]. Las diferencias en la capacidad antiviral mostrada por los sueros en estas especies, son un indicativo de la necesidad de evaluar la actividad antiviral intrínseca en sueros de diferentes especies domesticas susceptibles

naturalmente a este virus. Es posible que alguna especie domestica con menor actividad antiviral a la mostrada por el suero de cerdo en esta tesis, pudiera jugar un papel como amplificador del virus, siendo una fuente para los vectores de esta enfermedad en condiciones naturales.

Evaluación *post mortem*

El uso de la qPCR, nos permito detectar diferencias significativas en el perfil biodistribución encontrado en los tejidos colectados post mortem entre los diferentes grupos de cerdos infectados en este trabajo. En este sentido, es importante resaltar la mayor cantidad de ARN viral encontrada en los diferentes nódulos linfáticos colectados de los cerdos infectados con el virus NJ0612NME6, lo que es consistente con el nivel de virulencia más elevado observado en esta cepa viral.

Por otro lado, más allá de las diferencias en la cantidad de copias de RNA diferenciando cada uno de los grupos de cerdos, el perfil de biodistribución encontrado en ambos grupos, es consistente con la habilidad del virus para diseminarse de manera sistémica, lo que sería sugestivo a la existencia de una fase de viremia durante la infección del cerdo con el VSNJV.

Así mismo, este perfil de biodistribución podría soportar la idea de que, el VSV podría tener fases de replicación en otro tipo de tejidos más allá de los epiteliales, por lo que, el virus podrá estarse excretando por otras fuentes alternas a las lesiones vesiculares, como por ejemplo la orina, heces y semen. Al menos en el caso de heces fecales, un estudio previo en cerdos mostró la factibilidad para aislar el virus de esta muestra biológica [61]. En base a lo mencionado anteriormente, proponemos la importancia de la realización de trabajos futuros de investigación, con la finalidad de evaluar con más detalle, el papel que diferentes órganos (pulmones, corazón, intestinos, bazo, hígado, vejiga, riñones, vasos sanguíneos, así como diversos nódulos linfáticos asociados con estos tejidos) podrían estar jugando en la patogenia de esta enfermedad, durante la fase de infección aguda en el cerdo.

Finalmente, con base al perfil de biodistribución y a la propiedad de la qPCR para detectar la presencia de ARN viral en tejidos de cerdos infectados por este virus por lo menos veintiún

días posteriores a la infección, es posible proponer que, estos resultados podrían sustentar la elaboración de futuros planes de vigilancia de esta enfermedad en México y los Estados Unidos, mediante el monitoreo de viral a partir de muestras de tejidos porcinos colectadas en rastros y evaluadas por qPCR. También, esta prueba molecular podría ser usada para mejorar el diagnóstico de esta enfermedad, al permitir la detección de ARN viral proveniente de muestras de hisopos nasales y orales de cerdos afectados por esta enfermedad, donde no es posible colectar lesiones vesiculares, ofreciendo una alternativa para el diagnóstico de esta enfermedad.

Evaluación de la respuesta inmune.

Una vez establecidas las diferencias en los niveles de virulencia entre ambas cepas virales, se determinaron posibles diferencias en la respuesta inmune entre los grupos de cerdos infectados con cada uno de los dos virus, con la finalidad de generar una hipótesis y proponer una posible explicación al mayor nivel de virulencia mostrado por el virus epidémico NJ0612NME6. Basado en la diferencia en los niveles sistémicos de IFN1, TNF, IL-6 y la presencia de anticuerpos del tipo IgM entre los grupos de cerdos infectados con cada uno de los virus, es posible proponer que un posible mecanismo ligado a la virulencia del virus epidémico NJ0612NME6, el cual podría estar relacionado con su habilidad para interferir con la respuesta inmune innata en el cerdo, lo cual habría significado una ventaja adaptativa en la evolución de este fenotipo epidémico, al incrementar su nivel virulencia en el huésped vertebrado, elevando así los niveles de excreción viral y favoreciendo su trasmisión durante el evento epizoótico que involucro a este virus en México y los Estados Unidos.

En este sentido, nuestros resultados concuerdan con un estudio previo que muestra la función relevante que el IFN1 tiene en la respuesta antiviral durante la patogenia del VSV en el cerdo, al producir la atenuación de este virus [83]. De igual forma, los resultados mostraron que existe una relación negativa entre la inducción de interferón sistémico y los niveles de virulencia de los virus utilizados en este estudio. Asimismo, basándonos en las múltiples funciones inmunológicas en la que el IFN1 ha sido implicado [94], es posible especular que las diferencias de inducción sistémica de IFN1 durante la etapa aguda de la infección observadas entre

ambos grupos de cerdos, podrían haber influenciado los niveles de virulencia a tres diferentes niveles:

a) A nivel local previendo la presentación de lesiones vesiculares y consecuentemente afectando la excreción viral, mediante la inducción de un estado antiviral en los tejidos epiteliales, esto ocasionado por secreción de diferentes proteínas antivirales relacionados con la expresión de genes estimulados por el interferón. En este sentido, diferentes proteínas producidas por genes estimulados por acción del IFN1 han sido descritas con acción antiviral contra el VSV incluidas: BST2[95], GBP1 y GBP2[96], IFIT2[97], ISG20[98], y MX2[99].
b) Modulando la respuesta inmune innata al estimular la secreción de citoquinas y quimosinas proinflamatorias en macrófagos y células dendríticas, estimulando la presentación antigénica y presentación de respuestas Th1, así como estimulando la actividad de las células NK[100]. Estas últimas han sido demostrado tener un papel importante en el control de VSV [101].

   Asimismo, la propiedad del IFN1 para estimular la producción de citoquinas proinflamatorias [101], podría ayudarnos a explicar los niveles más elevados de a nivel sistémico de TNF observado en el grupo de cerdos infectado con el virus NJ0806VCB. En este contexto, investigaciones recientes muestran la retroalimentación positiva que existe entre TNF e IFN1, siendo su acción sinérgica esencial para promover la respuesta inflamatoria [102, 103]. Esto es interesante, ya que podría explicar el proceso de inflamación severo observado en las articulaciones de las patas de los cerdos infectados con el virus NJ0806VCB. De igual manera, es por demás relevante mencionar la acción antiviral creada por el TNF contra el VSV en cultivos previamente estimulados con esta citoquina [104]. Este fenómeno es explicado por la habilidad del TNF para estimular la transcripción del clúster de genes estimulados por el INF (ISG) de manera independiente al clásico mecanismo descrito para el IFN1 asociado con la cascada JAK-STAT [105], ofreciendo así otra posible explicación a los niveles más elevados de virulencia observados en los cerdos inoculados con el virus NJ0612NME6.

c) Modulando la respuesta inmune adaptativa, por la acción que el IFN1 tiene en la activación de las células B [94], lo que podría ayudar a explicar la aparición más temprana de anticuerpos IgM en el grupo de cerdos infectados con el virus NJ0806VCB.

Aunque en este punto no se cuenta con una explicación plausible sobre el mecanismo por el cual el virus NJ0612NME6 fue más eficiente para reducir la inducción sistémica de IFN1, con base a la evaluación transcriptómica en células primarias de macrófagos porcinos llevada a cabo en esta investigación, se puede hipotetizar que la mayor eficiencia de este virus para suprimir la trascripción del factor de regulación del interferón siete (IRF7) en estas células, podría ser un indicativo sobre el posible mecanismo de acción usado por este virus, para disminuir la inducción sistémica de IFN1 y en consecuencia ocasionar un aumento en los niveles de virulencia de este virus. Esta propuesta, seria consistente con en el mecanismo de supresión planteado para la cepa Norte Americana del virus del síndrome reproductivo y respiratorio porcino, en el que este virus es capaz de suprimir la trascripción de IRF7 en células dendríticas plasmocitoides porcinas (una de las principales fuentes celulares de IFN1 durante la infección viral), produciendo así un decremento en la producción de IFN1 en las células infectadas [106].

En este sentido, es importante considerar que, IRF7 es un factor de transcripción esencial para la inducción del IFN1(tipo α), el cual es expresado de manera constitutiva en células dendríticas plasmocitoides, células B y monocitos en el bazo, timo y linfocitos de la sangre periférica [107]. En los experimentos *in-vitro* llevados a cabo en esta tesis no fue posible demostrar diferencias en la replicación entre ambos virus, por lo que es posible proponer que evaluaciones adicionales en diferentes células de origen mieloide y linfoide, podrían ayudar a obtener conclusiones más sólidas sobre los mecanismos que podrían estar influenciado las diferencias entra ambas cepas para inducir de manera sistémica la producción de IFN1.

Otro resultado interesante encontrado en esta investigación durante la evaluación de respuesta inmune, fueron las diferencias en los niveles sistémicos de IL-6 mostradas entre ambos grupos de cerdos durante la fase aguda de la infección. Es interesante que no obstante

de los niveles sistémicos más elevados de IFN1 y TNF en el grupo de cerdos infectados con el virus NJ0806VCB, en el caso de la IL-6 los niveles sistémicos más elevados de esta citoquina se encontraron en los cerdos infectados con el virus NJ0612NME6, sugiriendo que los niveles sistémicos de IL-6 podrían correlacionar positivamente con la virulencia de las cepas de campo del VSNJV en el cerdo.

En este sentido, es interesante considerar los efectos contrastantes descritos durante la respuesta inmune contra los virus asociados a la acción de la IL-6 (Capitulo 7). La IL-6 es una citoquina pleiotrópica con una función dual, la cual está relacionada con la activación y el control de la respuesta inflamatoria durante la respuesta inmune a virus [108]. Evidencia experimental obtenida mediante la utilización de ratones transgénicos, indica que la acción de la IL-6 es fundamental en la respuesta antiviral a la infección con el VSV al estimular la producción de anticuerpos IgG durante la infección [109]. Asimismo, en el caso de virus de influenza la IL-6 ha sido implicada en la regulación optima de la respuesta de células T, así como previniendo la apoptosis en las células infectadas en el pulmón, previniendo así el daño ocasionado durante la infección por este virus [110, 111].

Sin embargo, clinicamente la acción de la IL-6 ha sido correlacionada positivamente con exacerbaciones de cuadros clínicos asociados a infecciones persistentes ocasionadas por otros virus RNA como son: virus de Andes [112], influenza [113], hepatitis B [114] y C [115], inmuno deficiencia humana [116], fiebre hemorrágica de Crimean-Congo [117], y Chikungunya [118]. En este sentido múltiples mecanismos asociados al incremento desmedido de la IL-6, incluyendo la reducción de la respuesta Th1, la reducción de la citólisis y apoptosis han sido documentados experimentalmente durante la respuesta innata contra diferentes virus RNA [108]. Aunque en este punto la explicación más probable al nivel más elevado de IL-6 detectado en los cerdos infectados con el virus NJ0612NME6, podría estar relacionado con los niveles más elevados de replicación de este virus en el cerdo, futuras investigaciones utilizando animales transgénicos podrían ser útiles para esclarecer el papel de esta citoquina como promotor de la virulencia del VSNJV.

Creación de una clona de cDNA del VSNJV.

Uno de los logros más importantes durante esta investigación, fue la creación de una clona de cDNA del VSNJV cepa epizoótica NJ0612NME6 [84] (capitulo 8), la cual fue utilizada para manipular el genoma del VSNJV y crear así por mutagénesis dirigida la mutante M51R la cual fue utilizada para obtener más información sobre la modulación de la respuesta inmune innata del VSNJV como factor de virulencia durante la infección en el cerdo.

La clona conteniendo el genoma completo de la cepa NJ0612NME6, y tres plásmidos de soporte conteniendo los genes implicados en la replicación (N, P, L), fueron ensamblados por el método de recombinación de sitio-específica utilizando la polimerasa purificada el virus de vaccinia [12]. Esto significó no sólo la validación de este método para el ensamblaje del genoma del VSNJV, sino una mejora en la relación en los métodos publicados anteriormente para la creación de las clonas de VSV [119, 120]. Esta mejora se debe a que el método recombinación del sitio especifica no es dependiente de sitios de restricción, lo que se traduce en una reducción de tiempo y una mayor eficacia durante el proceso de clonación.

De manera interesante, la evaluación del virus infeccioso obtenido a partir de la clona NJ0612NME6 conservó las características fenotípicas de virulencia mostradas por el virus parenteral durante su evaluación inicial en cerdos (capítulo 6), haciendo esta clona de cDNA ver una herramienta de trabajo importante para la conducción de futuros estudios involucrando genética reversa.

Modulación de la respuesta inmune innata por el VSNJV y la virulencia en el cerdo.

Finalmente, para obtener más detalles sobre la relación entre la habilidad del VSNJV para modular la respuesta inmune innata y la virulencia en el cerdo, el último trabajo de esta tesis doctoral se encamino en utilizar la mutante M51R como prueba de concepto para evaluar a más detalle la respuesta inmune innata contra el VSNJV durante la infección en el cerdo (capitulo 9).

La mutante M51R contiene una única substitución (metionina por arginina) en el aminoácido 51 de la proteína de matriz, la cual incapacita al VSNJV para suprimir de manera óptima la

respuesta inmune innata del hospedador, lo cual se traduce en un aumento no solo en la producción de IFN1, sino también en la de múltiples citoquinas y proteínas antivirales relacionadas con la respuesta inmune innata [19,20].

Los experimentos *in vitro* e *in vivo* llevados a cabo con la mutante M51R, produjeron resultados interesantes con relación a la interacción inmunológica del VSNJV con diferentes ambientes celulares (células de origen inmune y no inmune), y su relación con la virulencia en el cerdo.

In vitro, los estudios fueron llevados a cabo en dos tipos de cultivos primarios: células fetales de riñón de cerdo (FPKC) (representando células de origen no inmune), las cuales contienen una combinación de células epiteliales y fibroblastos [121], siendo un excelente modelo celular para evaluar el sitio primario de inoculación del virus en el ganado durante la infección por picadura de insecto en la naturaleza[1]. Por otro lado, como células de origen inmune se utilizaron cultivos primarios de macrófagos derivados de monocitos (PM-MQC). En este sentido, es importante mencionar el papel fundamental que los macrófagos juegan en la virulencia, al controlar la diseminación sistémica del VSV en el ratón [23,24].

De manera sorprendente, nuestros resultados mostraron la inexistencia de diferencias significativas en las cinéticas de crecimiento y formación de placas entre los virus NJ0612NME6 y la mutante M51R en FPKC, a pesar de la diferencia significativa en la producción de IFN 1 y algunos ISG observada en FPKC infectadas con el virus M51R. Contrastantemente, el virus M51R mostró menores niveles de replicación en comparación con el virus NJ0612NME6 durante las cinéticas de crecimiento llevadas a cabo en PM-MQC, lo cual fue consistente con la diferencia entre ambos virus en relación con expresión de genes asociados con los ISG.

A la luz de estos resultados, la diferencia entre FPKC and PM-MQC para afectar el crecimiento de la mutante M51R, podría ser explicado, debido a diferencias intrínsecas entre ambos fenotipos celulares. Mientras que los fibroblastos y células epiteliales son predominantemente productoras de interferón beta [94], los macrófagos expresan una gamma de receptores de reconocimiento de patrones moleculares de patógenos (receptores tipo toll), así como la expresión constitutiva de ISG como el IRF-7 (regulador positivo de diferentes

tipos de IFN1), lo que hace que estas células sean capaces de montar respuestas antivirales rápidas, reaccionado a concentraciones pico molares de IFN 1 [94, 107, 122] . Esta explicación es consistente con un estudio utilizando el virus de VSV Oncolítico VSV-IFNβ-NIS [83] , el cual tiene clonado dentro de su genoma, el gen humano que codifica para la producción del IFNβ [123], por lo que cada ronda de replicación el virus incrementa la concentración de IFNβ, produciendo así una acción autocrina y paracrina, previniendo la infección en las células vecinas, suprimiendo así la formación de placas y alterando el crecimiento del VSV-IFNβ-NIS en FPKC, produciendo un fenotipo opuesto al reportado en esta tesis con el virus M51R en las mismas células.

Los resultados sugieren que las células de origen inmune y no inmune representan dos retos diferentes para el VSNJV en relación con la evasión de la respuesta inmune innata durante la infección en el cerdo. En este sentido, esta investigación muestra que los PM-MQC son un fenotipo celular adecuado para la conducción de futuros estudios de expresión génica (microarreglos) encaminados a obtener más detalles sobre la virulencia del VSNJV en relación con la respuesta inmune innata contra el VSNJV en el ganado.

In vivo, los resultados confirmaron la inhabilidad mostrada por las células epiteliales *in vitro,* para montar una respuesta inmune innata optima contra el virus M51R, al no prevenir la formación de lesiones epiteliales en los sitios primarios de inoculación en los cerdos infectados con el virus M51R. Este resultado difiere con un estudio previo utilizando el virus oncolítico VSV-IFNβ-NIS, donde este virus fue incapaz de producir lesiones epiteliales en el sitio primario de inoculación en los cerdos [83]. Este es un concepto interesante, ya que, aunque ambos virus representan fenotipos virales con capacidad para incrementar la producción de IFNβ en las células infectadas, la presencia del gen del IFNβ en el genoma del virus VSV-IFNβ-NIS, permite que las células epiteliales puedan montar una respuesta inmune más rápida y eficaz ante la infección con este virus, en comparación a los que sucede con el virus M51R.

Con base a lo expresado anteriormente, y considerando la habilidad del virus M51R para producir lesiones vesiculares en los sitios de inoculación primaria de los cerdos infectados directamente, a pesar de su inhabilidad para suprimir adecuadamente la respuesta inmune innata en las células infectadas, sería posible argumentar que el tejido epitelial podría ser menos competente para montar respuesta inmunes adecuadas contra la infección del virus de VSNJV, por lo que la presencia de lesiones vesiculares en el sitio primario de infección, podría ser considerada como un signo clínico esperado en animales infectados naturalmente en el campo por el VSNJV, incluso cuando la infección haya sido producida por cepas con un menor grado de virulencia a la cepa a la cepa NJ0612NME6 utilizada en este estudio. Esto es interesante, ya que estudios de campo han mostrado que incluso la presencia de niveles significativos de anticuerpos neutralizantes detectados previamente a la infección con el virus de VSNJV, no son capaces de prevenir el desarrollo de lesiones vesiculares en animales infectados con este virus [124].

Por otro lado, *in vivo*, los resultados mostraron contrastantes diferencias clínicas entre los cerdos infectados con el virus M51R y los cerdos infectados con el virus NJ0612NME6, mostrando el profundo impacto que la mutación M51R produce en la virulencia del VSNJV en el cerdo. Este resultado junto con las diferencias contrastantes entre ambos virus para replicarse *in vitro* en PM-MQC, permiten especular de que, existe una correlación positiva entre la habilidad del VSNJV para replicarse macrófagos, con su capacidad para diseminarse de manera sistémicas en el cerdo, comprometiendo así la capacidad del VSNJV para producir lesiones epiteliales secundarias.

Esta hipótesis podría encontrar sustento en estudios llevados a cabo en ratones donde se muestra el papel esencial que juegan los macrófagos ubicados en los senos subcapsulares en los linfonodos, al prevenir la diseminación sistémica del VSV [125, 126, 127]. En este sentido se ha demostrado que posterior a la inoculación subcutánea del VSV, este es transportado vía linfática a los nódulos linfáticos regionales donde es fagocitado por los macrófagos ubicados en los senos subcapsulares, estos permiten la replicación del VSV, mediada intrínsecamente por el receptor tipo toll 7[127]. Este proceso esencial en la respuesta inmune contra el VSV, ya que este evento produce un aumento importante en la concentración de IFN 1, favoreciendo

así la activación de las células dendríticas plasmociticas residentes en el linfonodo, lo que conlleva a un incremento sustancial de IFN1 en el linfonodo y a una estimulación de la presentación antigénica en este órgano linfoide secundario, lo que previene la diseminación sistémica de este virus, indicando la importancia de los macrófagos en el proceso de control del VSV [125, 126, 127].

Lo descrito anteriormente nos permite sugerir que es necesario llevar a cabo trabajos futuros de investigación utilizando modelos de patogénesis temprana en animales domésticos, donde pueda evaluarse durante la etapa aguda de la infección, la interacción del VSNJV no solo con los macrófagos sino con las diferentes poblaciones celulares residentes en los linfonodos. En este sentido, llaman la atención los resultados obtenidos en el capítulo 3 de esta tesis doctoral, donde la evaluación de los órganos linfoides obtenidos durante las necropsias, muestran la presencia de una mayor concentración de RNA viral en los órganos linfoides evaluados de los cerdos infectados con el linaje epidémico 1.1 en comparación con los animales infectados con el linaje endemico1.2. Este resultado, aunado a la menor concentración de IFN1 sistémico en los animales encontrado infectados con el virus 1.1, seria posible proponer la hipótesis de que durante la etapa aguda de la infección, la mayor habilidad del virus asociado al linaje 1.1 para contrarrestar la producción de IFN 1 en los macrófagos residentes en las tonsilas, podría haber resultado en una mayor replicación viral y en un incremento en la diseminación sistémica, explicando el mayor número de lesiones epiteliales secundarias.

CAPÍTULO 11

CONCLUSIONES

I. Se demostró que la cepa epidémica del VSNJV NJ0612NME6 representa un fenotipo mas virulento para el cerdo, en comparación a su pariente endémico mas cercano la cepa NJ0806VCB.

II. Se demostraron las diferencias genómicas entre las cepas NJ0612NME6 y NJ0806VCB, indicando que diferentes proteínas en el genoma podrían ser responsables de las diferencias en virulencia entre ambas cepas.

III. Los resultados sugieren que la mayor virulencia mostrada por la cepa NJ0612NME6, podría estar asociado a la capacidad de este virus para regular la respuesta inmune innata en el cerdo.

IV. Se describe por primera vez la existencia de una fase de RNAemia durante la etapa aguda de la infección de VSNJV en el cerdo. De manera interesante, la intensidad de esta fase se correlacionó positivamente con las diferencias en virulencia entre ambas cepas.

V. Se construyó una clona de cDNA del virus epidémico NJ0612NME6, el cual constituye una herramienta muy importante para la conducción de trabajos futuros, relacionados a comprender el papel en la virulencia de sitios específicos en las diferentes proteínas del VSNJV.

VI. Se demostró que la respuesta inmune en las células epiteliales y en los macrófagos representan dos retos diferentes para el VSNJV durante la infección en el cerdo. En este sentido se identificaron a los macrófagos como un blanco para la realización de trabajos posteriores de investigación, encaminados a descifrar interacciones huésped-virus asociadas a la virulencia de VSNJV en el cerdo.

CAPÍTULO 12

PROSPECTIVAS

I. Determinar que las diferencias en virulencia entre las cepas NJ0612NME6 y NJ0806VCB demostradas en esta tesis en cerdos, son consistentes en otras especies domésticas (bovinos, equinos, caprinos, ovinos).

II. Realizar estudios similares utilizando otras cepas epidémicas y endémicas del VSNJV, para confirmar los resultados reportados en esta tesis.

III. Realizar estudios de patogenia temprana en el cerdo, para caracterizar a mayor de detalle el papel de diferentes órganos y tipos celulares en la infección con el VSNJV.

IV. Realizar estudios de genética reversa para caracterizar el papel que las diferentes mutaciones distintivas entre ambas cepas podrían jugar en la virulencia del VSNJV.

V. Se sugiere que, en estudios futuros de investigación de patogenia en cerdos, se aíslen células mononucleares de sangre periférica para intentar realizar aislamientos virales durante la fase de RNAemia en el cerdo y determinar así la existencia de una fase de viremia en la infección del VSNJV en cerdos.

VI. Realizar estudios de expresión génica en cultivos primarios de macrófagos porcinos para identificar genes relevantes durante la infección de estas células con el VSNJV.

VII. Estudiar a mayor detalle la respuesta inmune innata en células epiteliales ante la infección con el VSNJV.

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TRABAJOS GENERADOS DE ESTA TESIS

ARTICULOS EN REVISTAS INDEXADAS

Publicados:

1: **Velazquez-Salinas L**, Pauszek SJ, Verdugo-Rodriguez A, Rodriguez LL. Complete Genome Sequences of Two Vesicular Stomatitis New Jersey Viruses Representing the 2012 U.S. Epidemic Strain and Its Closest Relative Endemic Strain from Southern Mexico. Genome Announc. 2018 Feb 15;6(7). pii: e00049-18. doi:10.1128/genomeA.00049-18.

2: **Velazquez-Salinas L**, Pauszek SJ, Stenfeldt C, O'Hearn ES, Pacheco JM, Borca MV, Verdugo-Rodriguez A, Arzt J, Rodriguez LL. Increased Virulence of an Epidemic Strain of Vesicular Stomatitis Virus Is Associated with Interference of the Innate Response in Pigs. Front Microbiol. 2018 Aug 15;9: 1891. doi: 10.3389/fmicb.2018.01891. eCollection 2018.

3: **Velazquez-Salinas L**, Pauszek SJ, Barrera J, Clark BA, Borca MV, Verdugo-Rodriguez A, Stenfeldt C, Arzt J, Rodriguez LL. Validation of a site-specific recombination cloning technique for the rapid development of a full-length cDNA clone of a virulent field strain of vesicular stomatitis New Jersey virus. J Virol Methods. 2019 Mar; 265:113-116. doi: 10.1016/j.jviromet.2019.01.003. Epub 2019 Jan 9.

4: **Velazquez-Salinas L**, Verdugo-Rodriguez A, Rodriguez LL, Borca MV. The Role of Interleukin 6 During Viral Infections. Front Microbiol. 2019 May 10;10:1057. doi: 10.3389/fmicb.2019.01057. eCollection 2019.

5: **Velazquez-Salinas L**, Pauszek SJ, Holinka LG, Gladue DP, Rekant SI, Bishop EA, et al. A Single Amino Acid Substitution in the Matrix Protein (M51R) of Vesicular Stomatitis New Jersey Virus Impairs Replication in Cultured Porcine Macrophages and Results in Significant Attenuation in Pigs. Frontiers in Microbiology. 2020;11(1123).