A comparison of the effect of molluscum contagiosum virus MC159 and MC160 proteins on vaccinia virus virulence in intranasal and intradermal infection routes

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running title: The MCV MC159 protein decreases the virulence of vaccinia virus

Abstract

Molluscum contagiosum virus (MCV) causes persistent, benign skin neoplasm in children and adults. MCV is refractive to growth in standard tissue culture and there is no relevant animal model of infection. Here we investigated if another poxvirus (vaccinia virus; VACV) could be used to examine MCV immunoevasion protein properties *in vivo*. The MCV *MC159L* or *MC160L* gene, which encode NF-κB antagonists, were inserted into an attenuated VACV lacking an NF-κB antagonist (vΔA49), creating vMC159 and vMC160. vMC160 slightly increased vΔA49 virulence in the intranasal and intradermal routes of inoculation. vMC159 infection was less virulent than vΔA49 in both inoculation routes. vMC159-infected ear pinnae did not form lesions, but virus replication still occurred. Thus, the lack of lesions was not due to abortive virus replication. This system provides a new approach to examine MCV immunoevasion proteins within the context of a complete and complex immune system.

Molluscum contagiosum virus (MCV) is dermatotropic poxvirus, and is the etiological agent of molluscum contagiosum (MC) [1]. MCV infections are common and worldwide [2, 3]. MCV infects keratinocytes and infections can persist for months to years [4]. MC neoplasms are small and have little inflammation associated with them [1]. Lesions that spontaneously regress have increased numbers of apoptosing cells, cytotoxic T cells, natural killer cells, and type I IFN-expressing plasmacytoid dendritic cells [5]. Thus, it is presumed that one key to MCV persistence lies in MCV modulating the host response.

Very little is known about MCV immune evasion strategies as compared to other viruses. This is because MCV is refractive to growth in standard tissue culture. The sequencing of the MCV genome revealed that MCV encodes at least 40 known or predicted immune evasion molecules [6, 7]. Several of these proteins were characterized by studying them independent of MCV infection [4, 8-11]. However, it remains unknown how these MCV immune evasion molecules play a role in viral pathogenesis.

To overcome this technical barrier, we chose to deliver MCV immune evasion proteins (MC159 and MC160) to mice during a related poxvirus (VACV) infection. MC159 and MC160 were examined because each protein inhibits NF-κB activation, yet each uses different mechanisms to antagonize the NF-κB activation pathway [12-15]. We chose VACV because it is the best-studied poxvirus [16], and there are several excellent animal models of VACV infection that afford studying the impact of viral immune evasion molecules on viral pathogenesis [17-20]. Additionally, we published that MC159 and MC160 inhibit NF-κB and IRF3 activation in murine cell lines [21]. Further, Randall et al show that MC159 interacts with murine NEMO [15]. We also have unpublished data showing that MC160 induces murine IKK1 degradation, similar to MC160's effect on human IKK1 [13, 14]. Thus, even though MCV is a human pathogen, it is likely that MC159 and MC160 interact with some of the known equivalent murine binding partners involved in immune surveillance.

VACV strain vΔA49 was used as the parental virus, Fig. 1(a). vΔA49 lacks A49, which is an NF-κB antagonist [22]. We chose this virus because vΔA49 is moderately decreased in virulence [22], and because MC159 and MC160 are reported to inhibit NF-κB activation [12, 14, 15]. Furthermore, MC159 and MC160 each act upstream of A49 [12, 14, 15, 22]. Thus, insertion of MC159 or MC160 into vΔA49 might restore vΔA49 virulence.

The *MC159L* and *MC160L* genes were stably inserted into vΔA49, a virus construct that deleted nucleotides 113-473 of the *A49R* gene [22], Fig. 1(a). The expression of *MC159L* and *MC160L* was controlled by the VACV p7.5 promoter to ensure MCV gene expression throughout VACV infection. Of course, the expression profile for these MCV genes in VACV may differ from that of its profile during a natural MCV infection. The creation of vMC159 was described previously [12]. The strategy for constructing vMC160 was similar.

To create vMC160, an *MC160L* gene under the control of the VACV p7.5 promoter was inserted into the pΔA49MCS plasmid [12]. pΔA49MCS contains a mutated *A49R* gene lacking nt 113-473 that is flanked by a portion of the *A48R* and *A50R* genes [12]. The A48 flank begins at *A48R* nt 457 and continues through to *A49R* nt 112 for a 338-bp product [12]. It also possesses multiple cloning sites for insertion of the *MC160L* gene [12]. To create MC160/pΔA49MCS, the *MC160L* gene was PCR amplified from MC160/pCI [13] using forward primer 5'-
GGATCTATAATC<u>ATG</u><u>GCGCACGAGCCA</u> -3' and reverse primer 5'-
CTAG*ACTAGT*<u>CTAGTAGGAAGCTTTCGTT</u>-3'
to yield a 1212-bp PCR product. The MC160 nucleotides are underlined. The reverse primer for MC160 engineered in the *Spe* I restriction enzyme digestion site (italicized). The p7.5 promoter was PCR amplified from pUC13/gpt/EGFP [22], yielding a 121-bp product. The forward primer was 5'-TTTT*ATCGAT*<u>TAAATAATAAATACAATAATTAATTTCTCGT</u>-3' and the reverse primer was 5'- **CTCGTGCGCCAT<u>GATTATAGATCCG</u>**<u>TCACTG</u>-3'and this yielded a 121-bp PCR product. The forward primer introduced a *Cla*I restriction enzyme site (italicized).

Next, 50-100 ng of the gel-purified PCR products were joined by SOE, and PCR amplified using the A48R forward and A50R reverse primers described in Biswas et al [12]. The resultant PCR product (1433 bp) was digested with *Cla*I and *Spe*I and inserted into pΔA49MCS that had been digested with *Cla*I and *Spe*I and treated with SAP. This plasmid was named MC160/pΔA49MCS.

To create vMC160, CV-1 cells were infected with vΔA49 and transfected with pMC160 and transient dominant selection was used, as described previously [23]. Recombinant viruses were collected 24 h later selected in the presence of mycophenolic acid, xanthine and hypoxanthine [23]. This process was repeated three times to isolate recombinant viruses away from parental viruses. Intermediate EcoGPT^{+} viruses were resolved into vMC160 by plaquing on BSC-1 cells in the absence of drugs.

As shown in Fig. 1(b), the genotype of vMC160 was confirmed by using PCR analysis. Note that the 362-bp amplicon from vΔA49-infected cells increased in size to 1,692 bp, reflecting insertion of the 883-bp and 1,333-bp *MC160L* insert. Similarly, there was an 883-bp product when PCR amplifying the region flanking *MC159L*, as expected. Finally, the 55-kDa MC160 protein expression was detected in infected cells as early as 2 h (post-infection) p.i. and remained detectable at 24 h p.i. using polyclonal antiserum specific MC160 [24], Fig. 1(c). As previously reported, the 31-kDa MC159 protein was also detected using anti-MC159 antiserum. The VACV E3 protein, an early protein, was detected throughout infection, as predicted [25]. Actin levels were similar in each lane, showing even protein loading.

Intranasal (IN) inoculation allows examination of VACV virulence; VACV initially infects the lungs and then spreads to the brain and other distal organs [17, 26]. BALB/c mice were inoculated IN with 5 X 10^3 p.f.u. of a virus as described [27]. Mice were examined daily and we used a 5-point scoring system that determines the extent of illness [28, 29]. This was performed as a blinded study to minimize potential bias. We only analyzed the planned comparisons of

vΔA49 to either vMC159, vMC160 or vΔA49rev, and set *P* values of <0.05 (indicated by one asterisk), *P* <0.01 (as indicated by two asterisks) and *P* < 0.0001 (as indicated by 4 asterisks) as criteria for statistical significance for these and the remaining assays involving mice.

PBS-inoculated mice showed no signs of illness at any time point. vΔA49rev, which is equivalent to wild type VACV strain WR, triggered clinical signs of illness similar to results reported previously [22] (Fig. 2A). vΔA49 infection delayed illness onset, and clinical scores were consistently lower than vΔA49rev-infected mice as noted previously [22]. These differences were statistically significant on days 7-13 p.i. (Fig. 2A). vMC159 infection appeared to cause a milder disease; the clinical scores from vMC159-infected mice were lower than vΔA49-infected mice from days 8 -12 p.i., and these differences were statistically significant on days 8, 9, and 11-13 post-infection, Fig. 2(a). In comparison to vΔA49 infection, mice infected with vMC160 had increased signs of illness, Fig. 2(a). These differences were statistically significant at days 7 and 8 post-infection, Fig. 2(a). These data suggest that the *MC159L* gene reduced the virulence of vΔA49 while *MC160L* partially substituted for *A49L*.

Weight loss is an additional measure of virus virulence for IN inoculations [30]. The same mice as in Fig. 2(a) were also weighed daily, and data were expressed as the percentage of the mean of each individual animal's weight loss from day 0 +/- SEM [27]. Results are shown in Fig. 2(b). We only analyzed the planned comparisons of vΔA49 to either vMC159, vMC160 or vΔA49rev. vΔA49rev infection caused weight loss similar to that reported previously [22]. vΔA49-infected mice lost less weight than vΔA49rev-infected mice as expected [22], and these differences were statistically significant on days 8-12 p.i., Fig. 2(b). vMC159 caused less weight loss than vΔA49 at days 8-15 p.i. although these differences in weight were not statistically significant. vMC160-infected mice also weighed slightly more than vΔA49-infected mice at days 8-12 p.i., but these differences were not statistically significant. These data suggested that MC159 decreased virus virulence to a greater extent than vMC160 in the IN infection model.

152 The intradermal (ID) inoculation of a mouse ear pinna provides an alternative model to
153 examine VACV virulence [19, 31]. In this case, lesion formation and lesion size is used to
154 quantify virulence [18]. One could argue that ID inoculations most closely represent the location
155 of natural, dermatotropic MCV infections. There also are some parallels in the immune
156 responses to ID VACV infections and MCV infections. For example, type I IFN appears to be
157 important in controlling lesion size in VACV-infected ears [32] and lesion resolution during MCV
158 infections [5].
159 For ID inoculations, C57BL/6 mice were inoculated ID in the left ear dorsal pinna with
160 10^4 PFU of each virus as described in [27]. The infected ears were examined daily for the next
161 18 days for the presence of lesions, and results are shown in Fig. 3(a). We only analyzed the
162 planned comparisons of vΔA49 to either vMC159, vMC160 or vΔA49rev. For all virus infections,
163 no lesions were visually detected for the first six days p.i., a routine observation [27]. Gross
164 lesions were observed in vΔA49rev-infected mice starting on day 7 p.i., and lesion size
165 increased until day 12, and then resolved from days 13-18. vΔA49-induced lesion sizes were
166 slightly smaller than vΔA49rev at all times examined, and were statistically significant only on
167 days 10 and 11 p.i. vMC160-associated lesions were similar in size to lesions produced by
168 vΔA49rev and slightly larger that vΔA49-induced lesions. When comparing lesions from
169 vMC160- versus vΔA49-infected mice, vMC160 lesions were significantly larger on days 7,8 and
170 10 p.i., indicating that MC160 may increase virulence partially. Surprisingly, vMC159 inoculation
171 did not produce a lesion at any point in time, Fig 3(a) and (b).
172 The most striking results observed were those for the ID model of infection, and that
173 there was a complete lack of lesion formation during vMC159 infection. Only 2 other VACV
174 strains have been reported to not cause lesion formation in skin: vΔA36R and NYVAC [18, 33].
175 vΔA36R and NYVAC cannot spread from cell-to-cell efficiently *in vitro* [18, 33, 34]. It is thought

176 that ΔA36R or NYVAC do not cause lesions because they spread less efficiently to neighboring
177 cells *in vivo*, resulting in an abortive infection process.
178 One question was why vMC159 would cause no lesions. vMC159 and vMC160 each
179 replicated to the same levels as vΔA49 using either one-step or multi-step growth curve assays
180 in mouse embryo fibroblasts (MEFs) (data not shown). Also, vMC159- and vMC160-formed
181 plaques were similar to vΔA49 and vA49 in MEF and BSC40 cellular monolayers (data not
182 shown). Thus, it was unlikely that vMC159 spreads less efficiently for reason suspected for
183 vΔA36R and NYVAC. Another possibility was that MC159 and MC160 may not interact with the
184 murine homologs of their binding partners. This is also unlikely because MC159 interacts with
185 murine NEMO [15] and MC160 interacts with murine IKK1 (data not shown). Next, viral titers
186 were quantified at 3, 5 and 11 days p.i. [27]. Maximum VACV titers are detected at day 5 p.i.
187 [19], and we chose these time points to detect virus replication prior to and after maximal
188 replication. vMC160 titers were not examined because vMC160-induced ear lesions were
189 similar to those for vΔA49 and vΔA49rev.
190 Data are shown in Figure 3 as both p.f.u. per ear Fig. 3(c) and PFU per gram of tissue
191 Fig. 3(d). The starting inoculum was 10^4 p.f.u. in each ear pinna [27]. Data showed that all
192 viruses replicated because virus titers were higher than the initial inoculum (10^4 p.f.u.) on days 3
193 and 5 p.i. vMC159 titers were lower than the wild-type (vA49rev) or parental (vΔA49) viruses at
194 all times tested. vΔA49 and vΔA49rev titers increased to approximately 1.3 x10^6 by day 3 p.i.
195 (Fig. 3C). In contrast, vMC159 titers were 1.2 x 10^5 p.f.u. at day 3 p.i. All viruses continued to
196 replicate during the next 48 h because titers increased between 3 and 5 days p.i. By day 11 p.i.,
197 virus replication had waned, as indicated by the decrease in virus titers. Note that the decrease
198 in virus titers from day 5 to day 11 p.i. implies that there is indeed immune mediated clearance
199 of virus, but this occurred without accompanying inflammation.

One could argue that no lesions arise because vMC159 titers are lower than vΔA49 in ear pinnae. Indeed, it is unclear what the threshold of virus titer is needed for lesion formation. Tscharke *et al.* showed that inoculation with as little as 10^2 p.f.u. of VACV induces ear lesions formation [18]. vMC159 titers greatly exceed that amount at days 3 and 5 p.i. This suggests that vMC159 decouples replication from lesion formation. Interestingly, both ΔA36R and NYVAC elicit protective immune responses (e.g., antibody production) [18, 35]. Thus, it is tempting to ask if vMC159 also retains its immunogenicity and, if so, if vMC159 would be useful for the vaccine field.

It is not yet clear how MC159 suppresses lesion formation, and this is a direction for future studies. It is appreciated that VACV lesions are due, in part, to immunopathology because smaller lesions are associated with decreases in expression of multiple cytokines and chemokines [27, 36]. Thus, MC159 may either directly or indirectly prevent expression of these host cell proteins to halt pro-inflammatory processes. In this case, the MC159 ability to inhibit NF-κB and IRF3 activation may be relevant. MC159 inhibits apoptosis while MC160 does not [24, 37]. Another speculation, then, is that this anti-apoptotic property of MC159 affords virus attenuation, perhaps by allowing virus-infected cells to survive for prolonger time periods. Interestingly, a mutant VACV lacking an apoptosis antagonist (ΔB13R) has an increased lesion size as compared to wild-type VACV [18], showing an instance where inhibition of apoptosis diminishes lesion formation.

This is the first report that examines MCV immune antagonists in the context of an animal infection. MC159 and MC160 are members of the FLIP family of proteins [4]. They each possess tandem death effector domains (DEDs) that share 43% similarity. MC159 and MC160 also share some biological features, including inhibition of NF-κB and IRF3 [4]. Despite their similarity, MC159 and MC160 likely have distinct roles during MCV infection *in vivo*, as indicated by data here. VACV itself expresses at least 10 different NF-κB inhibitory proteins [20],

indicating that control of this pathway is critical for survival of VACV *in vivo*. Perhaps MCV also expresses MC159 and MC160, along with the two other known MCV NF-κB antagonists (MC005, MC132), for similar reasons when confronting a complex, multi-faceted anti-viral immune response [10, 11].

To date, there is no cell culture system or laboratory animal model to study MCV replication and pathogenesis. Researchers have used creative approaches to extrapolate the biological importance of MC159 as an immune evasion molecule. This includes the development of transgenic mouse strains that express MC159 [38, 39] or using murine cytomegalovirus to express MC159 [40]. Our system uses VACV during ID mouse infections, and is perhaps the model closest to mimicking MCV infection at this current time. One could argue that the addition of MC159 to vΔA49 resulted in a disease that mimics MC because, like MC, there is little inflammation.

The current study demonstrates the biological effects of two well-characterized MCV immune evasion proteins in a newly-created system. MCV encodes at least 40 other known or predicted immune evasion molecules [6, 7]. Thus, studies of MCV immune evasion molecules provide a rich opportunity to identify novel aspects of virus-host interactions during persistent infections. The surrogate system described here now affords these types of studies to better understand MCV pathogenesis and persistent virus infections.

Figure Legends

Figure 1. Characterization of vaccinia viruses expressing either MC159 or MC160. (A) A schematic of the viruses used in this study, focusing on the portion of the VACV WR genome containing the *A49R* gene and portions of the *A48R* and *A50R* genes flanking *A49R*. vΔA49 is a VACV strain in which *A49R* nucleotides 113-473 are deleted. Either the MCV *MC159L* or the *MC160L* genes, each under the control of the VACV p7.5 promoter, were inserted into vΔA49 to create vMC159 or vMC160, respectively. U5NU is the early gene transcription termination signal. (B) BSC40 monolayers were either mock-infected or infected with the indicated viruses (MOI = 10). At 24 h p.i., cells were collected, and DNA was isolated. DNA was PCR amplified using a forward primer specific for *A48R* and reverse primer specific for *A50R* [22]. A portion of each PCR reaction was analyzed by gel electrophoresis. Bands were detected by ethidium bromide staining of the gel. (C) BSC40 monolayers were either mock-infected or with vΔA49, vMC159 or vMC160 (MOI = 10). Cells were lysed at the indicated times and 15 μg of clarified cellular lysates were subjected to immunoblotting for the presence of MCV (MC159, MC160) or VACV (E3) proteins or cellular β-actin.

Figure 2. The effect of MCV genes on virus virulence using the intranasal route of infection. Female BALB/c mice (n = 5 per group) were inoculated IN with 5 X 10^3 p.f.u. vΔA49, vΔA49rev, vMC159, or vMC160 or PBS. (A) Clinical signs of illness were monitored daily for 15 days and scored from 0-5. Clinical scores were expressed as the mean for each group. One-way ANOVA was performed followed by Tukey's multiple comparison test to determine statistical significance. Asterisks indicate the days on which clinical signs of illness induced were significantly different between the indicated groups. (*$P < 0.05$, **$P < 0.01$ or ****$P < 0.0001$). (B) Mice were weighed daily, and data were expressed as the percentage of weight change from day 0. To determine statistically significant differences between weight change during virus

infection, two-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test, was performed. Asterisks indicate the days on which weight change induced by vΔA49 were significantly different from those induced by vΔA49rev (*P < 0.05*).*

Figure 3. The effect of MCV genes on virus virulence using the intradermal route of inoculation. C57BL/6 mice (n = 5 per group) were infected ID with 10^4 p.f.u. vΔA49, vΔA49rev, vMC159, or vMC160 in the left ear pinna. Lesion size was expressed as the mean for the group +/- SEM. (A) Sizes of resulting lesions were measured daily for 18 days. The lesion size was measured by using a 0.01-mm digital caliper. The data are expressed as the means of lesion sizes ±SEM. Statistical significance was determined by two-way ANOVA, followed by Tukey's multiple comparison test. The asterisks indicate the days on which the lesion size caused was statistically significantly between indicated groups (*P < 0.05 or ****P < 0.0001). (B) Representative images of inoculated ear pinnae at 10 days p.i. vΔA49-infected mice (left panel) or vMC159-infected mice (right panel). (C and D) At the indicated days p.i., ears were collected, homogenized, and lysed, and viral titers of the lysates were determined by plaque assay. Each symbol represents the virus titer from an individual animal, and the mean titer is indicated by a line. Data are expressed as the mean titer of virus (p.f.u.) per gram of tissue (D) and as the total p.f.u. per ear (C). Statistical significance was determined by Kruskal-Wallis test. The asterisk indicate data points at which titers of viruses were statistically significantly different from the other (*P < 0.05).

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Ethical Statement

The work described was undertaken with ethical approval from the University of Illinois IACUC.

Conflicts of interest.

The authors declare that there are no conflicts of interest that they are aware of.

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Figure 1.

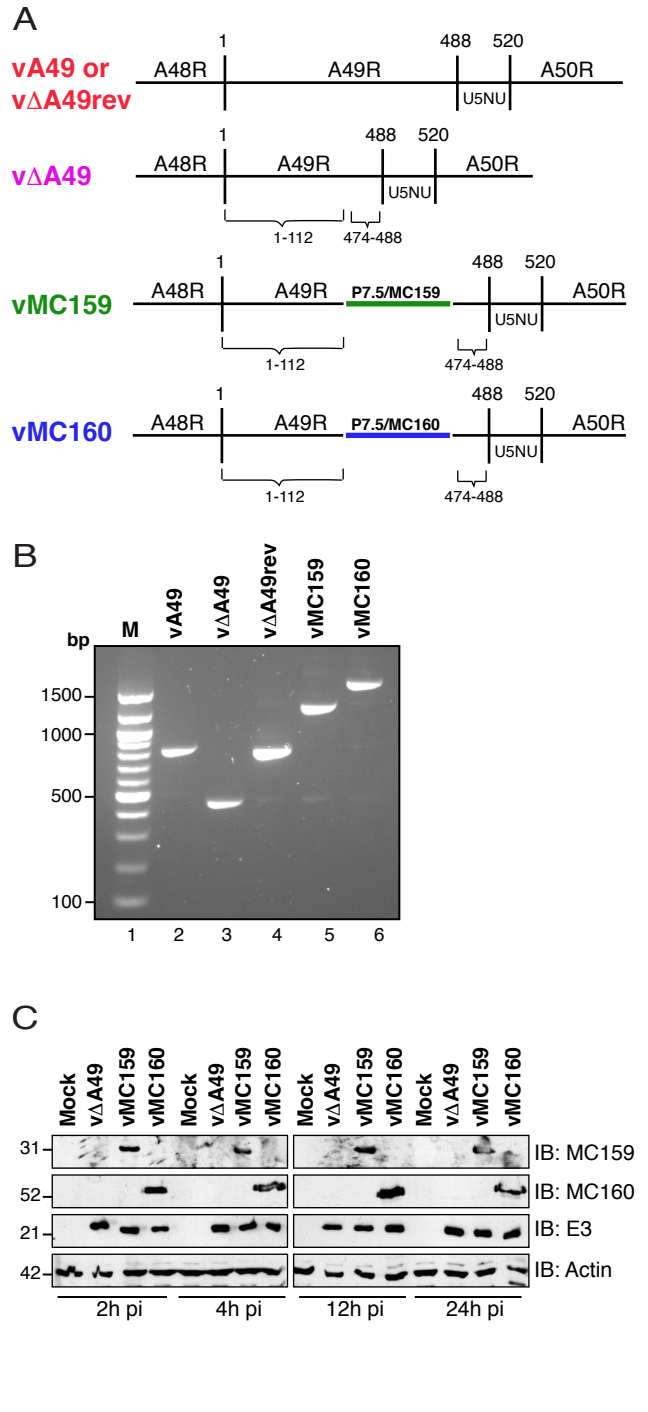
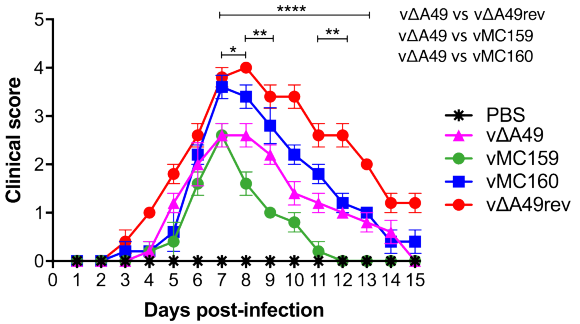

A
vA49 or
vΔA49rev
1
488
520
A48R
A49R
A50R
U5NU
vΔA49
1-112
474-488
vMC159
P7.5/MC159
vMC160
P7.5/MC160
B
M
vA49
vΔA49
vΔA49rev
vMC159
vMC160
bp
1500
1000
500
100
1
2
3
4
5
6
C
Mock
vΔA49
vMC159
vMC160
31
52
21
42
IB: MC159
IB: MC160
IB: E3
IB: Actin
2h pi
4h pi
12h pi
24h pi

Figure 2.

A


****
**
**
*
vΔA49 vs vΔA49rev
vΔA49 vs vMC159
vΔA49 vs vMC160
Clinical score
Days post-infection
PBS
vΔA49
vMC159
vMC160
vΔA49rev

B

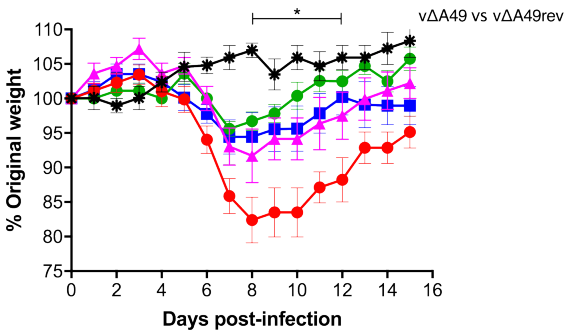
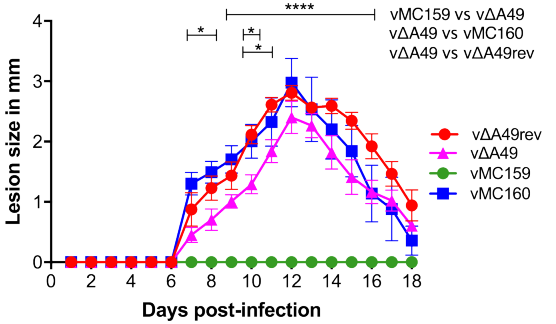

*
vΔA49 vs vΔA49rev
% Original weight
Days post-infection

Figure 3.

A



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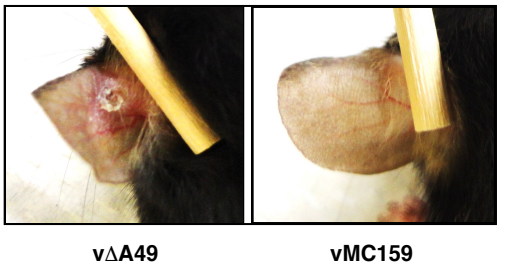
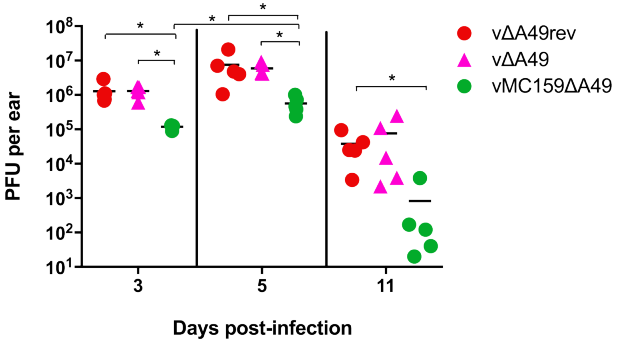


C



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