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SHORT COMMUNICATION

African swine fever virus *NL* gene is not required for virus virulence

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Previously, we described a highly conserved non-essential African swine fever virus (ASFV) right variable region gene, *NL*. Deletion of *NL* from the European pathogenic isolate E70 resulted in almost complete attenuation of the virus in domestic swine. To study gene function further, *NL* gene deletion mutants were constructed from two pathogenic African ASFV isolates, Malawi Lil-20/1 (Mal) and Pretoriuskop/96/4 (Pr4). Unexpectedly, both Mal (Mal-ΔNL) and PR4 (Pr4ΔNL) null mutants remained highly virulent when inoculated in swine. Mal-ΔNL exhibited a disease and virulence phenotype indistinguishable from its revertant, Mal-NLR, which caused 100% mortality. Mortality among Pr4ΔNL-infected animals was also high; however, a significant delay in onset of fever and viraemia and in time to death was observed. These data indicate that *NL* gene function is not required for ASFV virulence and that other yet-to-be identified viral determinants perform significant virulence functions in these African field isolates.

African swine fever (ASF) is a highly lethal and significant disease of domestic pigs, for which there is no vaccine or disease control strategy other than animal slaughter. The causative agent, a large enveloped double-stranded DNA virus (ASFV), is the sole member of the newly named *Asfarviridae* (Dixon *et al.*, 1995; L. Dixon, personal communication). ASFV genomic organization and its cytoplasmic replication strategy share similarities with the *Poxviridae* (Costa, 1990; González *et al.*, 1986; Ortin *et al.*, 1979; Sogo *et al.*, 1984).

ASFV is the only known DNA arbovirus (Brown, 1986; Costa, 1990; Dixon *et al.*, 1995). In nature, the perpetuation and transmission of this virus involve the cycling of virus between two highly adapted hosts, *Ornithodoros* ticks and wild pig populations (warthogs and bushpigs) in sub-Saharan Africa (Plowright *et al.*, 1969*a*, *b*; Thomson *et al.*, 1983; Wilkinson, 1989). In the warthog host, infection is subclinical, characterized by low-titre viraemias (Plowright *et al.*, 1994; Thomson *et al.*, 1980). Persistent infection of both ticks and pigs occurs following infection (Carrillo *et al.*, 1994; de Kock *et al.*, 1940; DeTray, 1957; Heuschele & Coggins, 1969; Kleiboeker *et al.*, 1998; Plowright *et al.*, 1969*a*; Sanchez-Botija,1963; Thomson *et al.*, 1983).

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In domestic pigs, ASF occurs in several disease forms, ranging from highly lethal to subclinical infections, depending on contributing viral and host factors (Colgrove *et al.*, 1969; Mebus *et al.*, 1981). ASFV infects cells of the mononuclear-phagocytic system, including highly differentiated fixed-tissue macrophages and reticular cells; affected tissues show extensive damage after infection with highly virulent virus strains (Colgrove *et al.*, 1969; Konno *et al.*, 1971*a*, *b*; Lord *et al.*, 1990; Mebus, 1988; Moulton & Coggins, 1968). ASFV strains of lesser virulence also appear to infect these cell types, but tissue involvement and resulting tissue damage are much less severe (Hess, 1981; Mebus *et al.*, 1981; Mebus, 1988). The abilities of ASFV to replicate and to induce marked cytopathology in these cell types *in vivo* appear to be critical factors in ASFV virulence. The nature of viral factors responsible for the differing outcomes of infection with strains of high virulence and of lesser virulence are largely unknown.

Previously, we described an ASFV gene, *NL*, with similarity to the neurovirulence gene *ICP34.5* of herpes simplex virus (Chou *et al.*, 1990; McGeoch & Barnett, 1991), a myeloid differentiation primary response gene, *MyD116* (Lord *et al.*, 1990), a growth arrest DNA damage gene, *GADD34* (Fornace *et al.*, 1989) and a partial ORF from *Amsacta moorei* entomopoxvirus (Hall & Moyer, 1991), and found it to be highly conserved among pathogenic ASFV isolates existing in either a long (*23-NL*, 184 amino acids) or short (*NL-S*, 70–72 amino acids) gene form (Sussman *et al.*, 1992; Zsak *et al.*, 1996). Deletion of *NL-S* from the highly virulent European isolate E70 resulted in marked attenuation of the virus in the swine host. Pig infections with the null mutant were characterized by a lack of clinical disease apart from a transient fever response, and by reduced viraemia titres (1000-fold) and no mortality (Zsak *et al.*, 1996). Notably, pigs previously infected with the E70 *NL-S* gene deletion mutant E70/43 were resistant to infection when subsequently challenged with fully virulent parental E70

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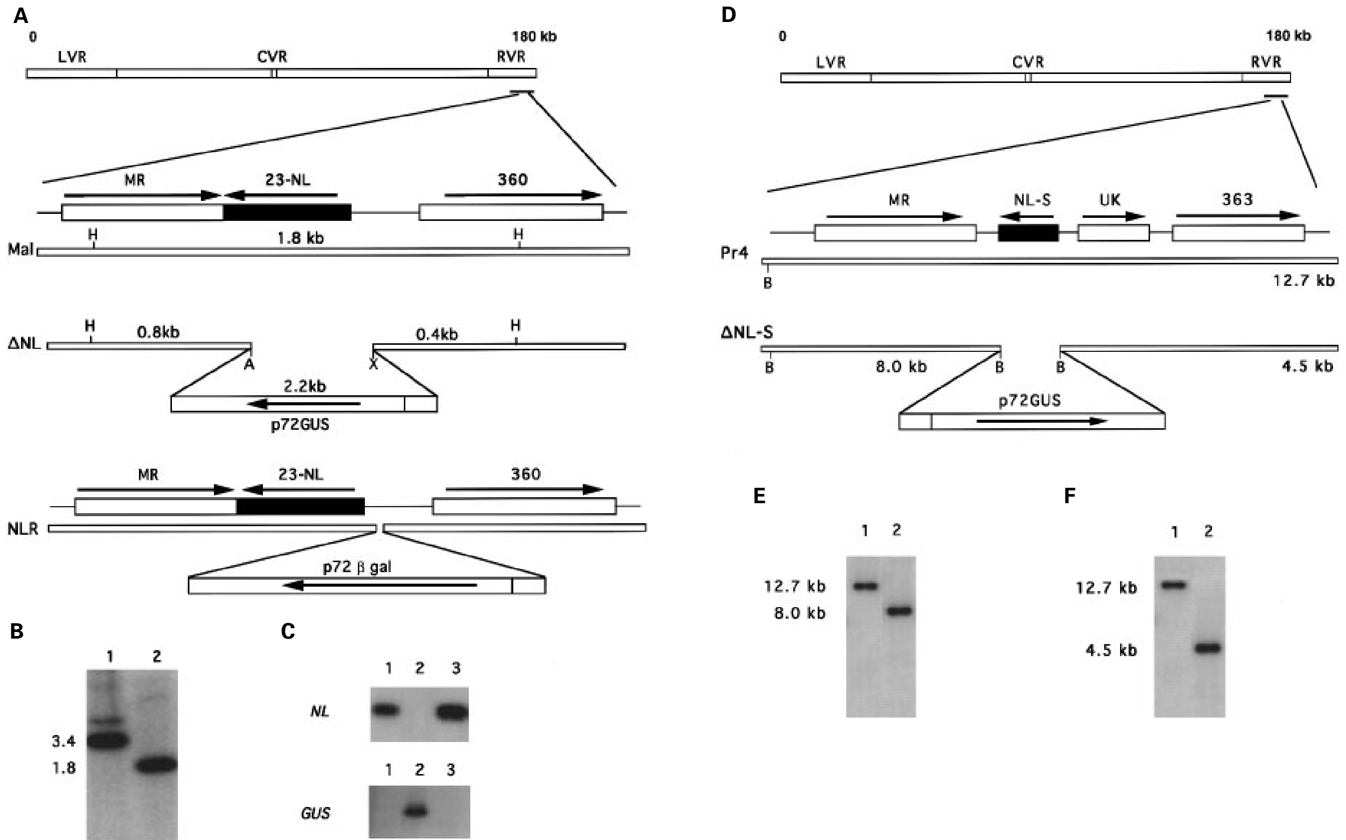

Fig. 1. (A-C) Characterization of a Mal *NL* gene deletion mutant, Mal-ΔNL, and its revertant, Mal-NLR. (A) Diagram of the *NL* gene regions in parental Mal, Mal-ΔNL and Mal-NLR. H, *Hin*dIII site. (B) Southern blot analysis of Mal-ΔNL (lane 1) and Mal (lane 2). Purified DNA was digested with *Hin*dIII, electrophoresed, blotted and hybridized to the parental 1·8 kbp *Hin*dIII fragment containing the *23-NL* gene and adjacent flanking regions. (C) PCR of *NL* and GUS sequences from Mal (lane 1), Mal-ΔNL (lane 2) and Mal-NLR (lane 3) viral DNA. (D–F) Characterization of an ASFV Pr4 *NL-S* gene deletion mutant, Pr4ΔNL-S. (D) Diagram of the *NL* gene regions in parental Pr4 and Pr4ΔNL-S. B, *Bam*HI site. (E) and (F) Southern blot analysis of Pr4 (lane 1) and Pr4ΔNL-S (lane 2). Purified DNA was digested with *Bam*HI, electrophoresed, blotted and hybridized to Pr4 DNA probes located at the left (E) and the right (F) of the engineered *NL* gene deletion. Positions of molecular size markers are indicated in kbp. LVR, left variable region; CVR, central variable region; RVR, right variable region.

virus (Zsak *et al.*, 1996). These observations suggested that ASFV *NL* gene deletion mutants might be suitable for use as engineered live attenuated ASFV vaccines.

In this report, we demonstrate that *NL* gene deletion mutants of two pathogenic African ASFV isolates, Malawi Lil-20/1 (Mal) and Pretoriuskop/96/4 (Pr4), remained highly virulent when inoculated in swine. Thus, *NL* gene function is not an absolute requirement for virulence in all ASFV isolates, and other yet-to-be identified viral determinants must perform significant virulence functions.

To examine further the significance of the ASFV *NL* gene for pig virulence, recombinant *NL* gene deletion mutants were constructed of two additional pathogenic African ASFVs isolated from ticks (Dixon, 1988; Haresnape *et al.*, 1988; Kleiboeker *et al.*, 1998), Mal, which contained the long *NL* gene form (184 amino acids) and Pr4, which contained the short form of the gene (71 amino acids). ASFV recombinant viruses were generated by homologous recombination between parental ASFV genomes and engineered recombination transfer vectors following transfection-infection in primary swine macrophage cell cultures as previously described (Neilan *et al.*, 1997; Zsak *et al.*, 1996).

The recombination transfer vector for introducing the *NL* gene deletion into Mal was prepared by inserting a reporter gene cassette containing the *β*-glucuronidase (GUS) gene with the ASFV late gene promoter (p72GUS) (Neilan *et al.*, 1997) into unique *Xba*I and *Apa*I sites located within a subcloned 2·85 kbp *Sph*I-*Sal*I fragment (pNL1) from the right variable region of Mal (Dixon, 1988). This deletion removed all but 108 5′-terminal nucleotides of the *NL* ORF (Fig. 1A). Adjacent upstream sequences of the p72GUS cassette and the remaining *NL* gene sequences do not contain a start codon, so expression of a truncated NL protein was not possible. Transfection-infection assays were done as previously described (Zsak *et al.*, 1996). A Mal *NL* gene deletion mutant, Mal-ΔNL (GUS-positive), was isolated, plaque-purified on macrophage cell

cultures and analysed by PCR and Southern blotting (Zsak *et al.*, 1996). A revertant of Mal-ΔNL was also constructed. A reporter cassette containing the *β*-galactosidase (GAL) gene with the p72 promoter (p72GAL), was inserted at the unique *Xba*I site of pNL1 (Fig. 1A). This construct was then used in transfection-infection experiments to restore the NL ORF in the Mal-ΔNL genome. A revertant virus Mal-NLR, a GUS-negative and GAL-positive virus, was purified by plaque assay and characterized as described above. Characterization of ASFV recombinants Mal-ΔNL and Mal-NLR is shown in Fig. 1(B–C). Viral DNA was purified from macrophage cell cultures infected with Mal, Mal-ΔNL and Mal-NLR as previously described (Wesley & Tuthill, 1984), digested with *Hin*dIII, separated by gel electrophoresis, Southern-blotted and hybridized to ^{32}P-labelled DNA probes. A novel *Hin*dIII fragment with a predicted size of 3·4 kbp was observed for Mal-ΔNL when probed with sequences flanking the *NL* gene region. The net 1·6 kbp size increase resulted from the introduced deletion of 652 bp together with the insertion of the 2·2 kbp p72GUS cassette (Fig. 1B, lane 1). Deleted *NL* gene sequences were not detected in Mal-ΔNL by PCR (Fig. 1C, lane 2). The predicted 147 bp *NL* PCR fragment was, however, observed in both parental (lane 1) and revertant (lane 3) viruses.

The recombination transfer vector for introducing the *NL* gene deletion into the Pr4 ASFV isolate was prepared as follows. Flanking DNA fragments mapping to the left (800 bp) and right (1024 bp) of *NL-S* were amplified by PCR using Pr4 genomic DNA as a template. Primer sets, each of which introduced a *Bam*HI site (underlined below) adjacent to *NL-S* were:

left flank forward primer
5′ TGCAAAATAAGATCTCAAATT 3′;
left flank reverse primer
5′ AGGGGATCCTATCCGCGGTGC 3′;
right flank forward primer
5′ CATGGATCCTGTGGGATTAGTGTT 3′;
right flank reverse primer
5′ GCACTTGTAGAGTGGATGGCA 3′.

The fragments were cloned into pCRII to give pTNL-S and were sequenced to verify ASFV sequences. The nucleotide sequences of the cloned flanking regions were identical over their entire length to the Pr4 genomic DNA sequence. The p72GUS reporter gene cassette was inserted into *Bam*HI-digested pTNL-S to yield p72GUSΔNL-S. This deletion removed all but the 5′-terminal 45 nucleotides of *NL-S* (Fig. 1 D). A Pr4 *NL-S* gene deletion mutant, Pr4ΔNL-S (GUS-positive), was prepared and analysed as described above. Pr4 and Pr4ΔNL-S viral DNA was purified from infected macrophage cell cultures, digested with *Bam*HI, Southern-blotted and hybridized to ^{32}P-labelled probes containing sequences which flanked the deleted region. The right-terminal *Bam*HI fragment of the Pr4 genome, which contained the *NL* gene region, was 12·7 kbp (Fig. 1E, F, lanes 1). Novel *Bam*HI fragments of predicted size 8·0 and 4·5 kbp were observed for Pr4ΔNL-S when probed with the two parental flanking fragments from this region (Fig. 1E, F, lanes 2).

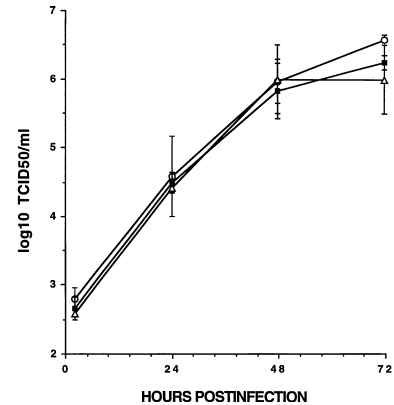

Fig. 2. Growth characteristics of ASFV Mal (○), Mal-ΔNL (■) and Mal-NLR (△) in infected swine macrophage cell cultures (m.o.i. 0·01). At indicated times, duplicate samples were collected and titrated for total virus yields. Data represent the means and standard errors of three or four independent experiments.

Growth characteristics of Mal-ΔNL and Pr4ΔNL-S were compared with those of parental and/or revertant viruses by infecting primary macrophage cell cultures (m.o.i. 0·01) and determining titres at various times post-infection as previously described (Zsak *et al.*, 1996). Growth kinetics and virus yields of Mal-ΔNL were indistinguishable from those of Mal and Mal-NLR (Fig. 2). Similarly, as previously shown for the E70 *NL-S* gene deletion mutant E70/43 (Zsak *et al.*, 1996), Pr4ΔNL-S exhibited parental growth characteristics on macrophages (data not shown). Thus, like the short gene *NL-S*, the longer *NL* gene, which encodes an additional 113 amino acids at the amino terminus of the protein, is nonessential for virus growth in swine macrophage cell culture.

To examine the virulence phenotype of Mal-ΔNL and Pr4ΔNL-S in domestic swine, Yorkshire pigs (30 to 35 kg) were inoculated intramuscularly with 10^2 $TCID_{50}$ null mutants or parental and/or revertant viruses. A dose of 10^2 $TCID_{50}$ Mal or Pr4 represents between 10 and 100 lethal doses (Neilan *et al.*, 1997). Clinical signs of ASF [fever (a rectal temperature of greater than or equal to 40 °C), anorexia, lethargy, shivering, cyanosis and recumbency] were monitored daily. Blood samples were collected every other day for the duration of the experiment. Virus isolation and titration of ASFV in blood samples were performed as previously described (Onisk *et al.*, 1994). Data from these experiments are shown in Table 1.

Unexpectedly, and unlike our prior observations for the E70 *NL* gene deletion mutant E70/43 (Zsak *et al.*, 1996), Mal-ΔNL remained highly virulent in domestic pigs. Apart from a modest reduction in mean viraemia titres of Mal-ΔNL infected animals, disease onset, disease course and mortality were similar for Mal-ΔNL and its revertant Mal-NLR (Table 1, Expt 1). Pr4ΔNL-S also remained virulent with an 86% mortality

Table 1. Swine survival, viraemia and fever response following infection with Mal-NLR, MalΔNL, Pr4 and Pr4ΔNL viruses

				Fever	Viraemia		
Expt	Group (*n*)	Number surviving	Days to death	Days to onset	Days to onset	Mean titre*	Maximum titre*
1	Mal-NLR (5)	0/5	9·8±0·4	4·6±0·4	3·6±0·4	7·6±0·1	8·0±0·1
	MalΔNL (5)	0/5	11·0±0·3	5·4±0·2	4·0±0·0	6·6±0·4[b]	7·8±0·2
2	Pr4 (3)	0/3	8·3±0·7	3·3±0·3	2·0±0·0	7·8±0·6	9·1±0·3
	Pr4ΔNL (3)	1/3	15·0±5·9[d]	6·0±1·1[a]	5·3±1·3[c]	6·3±1·0	7·4±1·0
3	Pr4 (4)	0/4	9·0±0·4	4·2±0·2	2·0±0·0	6·7±0·2	8·2±0·1
	Pr4ΔNL (4)	0/4	15·5±0·3[d]	4·7±0·5[a]	4·0±0·0[c]	7·2±0·2	8·0±0·1

Values significantly different from those of the Mal-NLR- or Pr4-infected group are indicated as follows: *a*, $P < 0{\cdot}04$; *b*, $P < 0{\cdot}03$; *c*, $P < 0{\cdot}007$; *d*, $P < 0{\cdot}0001$. Data from experiments 2 and 3 were pooled for statistical analysis.

* $\log_{10}$ haemadsorption units (HAD_{50})/ml (mean ± SE).

rate; however, aspects of virus infection were altered (Table 1, Expt 2 and 3). Significant delays in the onset of fever and viraemia (1 to 3 days) and in time to death (approximately 6 days) compared with parental Pr4-infected animals were observed. Mean and maximum viraemia titres were unaffected by the gene deletion.

These data indicate that *NL* gene function is not an absolute requirement for ASFV virulence and that the contribution of the *NL* gene to virus virulence is strain (isolate) dependent. For the three ASFV *NL* gene deletion mutants examined for pig virulence, there was a continuum of effect on virulence ranging from near complete attenuation (E70/43) (Zsak *et al.*, 1996) through delayed disease onset and death (Pr4ΔNL-S) to little or no effect (Mal-ΔNL). In the case of the two African ASFV isolates described here, a yet-to-be identified viral determinant(s) must perform significant virulence functions. This might involve an additional viral gene(s) capable of directly complementing the *NL* defect or alternatively, a viral gene(s) encoding a dominant virulence factor absent or inactive in the E70 isolate. In either event, the relative importance of *NL* for virus virulence may depend on the complement of other virulence and swine host range genes encoded by a given ASFV isolate.

Importantly, these data indicate that *NL* gene deletion alone is not sufficient to engineer live attenuated host range-restricted ASFV vaccines. Identification and further characterization of the virulence determinant(s) present in African isolates will be necessary.

These data also suggest that although still highly pathogenic, the European E70 isolate and perhaps other European pathogenic ASFV isolates may be more attenuated and/or adapted in domestic pigs than either of the two African viruses examined here. In nature, perpetuation of ASFV involves the cycling of virus between highly adapted hosts, *Ornithodoros* ticks and warthogs and bushpigs in sub-Saharan Africa (Plowright *et al.*, 1969*a*; Thomson *et al.*, 1983; Wilkinson, 1989), and it is under these conditions with their attendant pressures that virus fitness is selected for. The ASF E70 virus was isolated in Spain in 1970, 10 years after the introduction of virus into the Iberian peninsula (Tabarés *et al.*, 1987). During this period, transmission of virus largely occurred by spread within domestic pig populations. Therefore, it is possible, either due to the lack of selective pressure under these conditions or to virus-host adaptation, that the function of viral genes important in the natural cycle of infection may have been lost or altered. If this is the case, other findings on aspects of virus virulence and protective immunity against European ASFV isolates may not necessarily be applicable to African viruses found in nature.

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