Smallpox virus plaque phenotypes: genetic, geographical and case fatality relationships

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Smallpox (infection with Orthopoxvirus variola) remains a feared illness more than 25 years after its eradication. Historically, case-fatality rates (CFRs) varied between outbreaks (<1 to ~40%), the reasons for which are incompletely understood. The extracellular enveloped virus (EEV) form of orthopoxvirus progeny is hypothesized to disseminate infection. Investigations with the closely related Orthopoxvirus vaccinia have associated increased comet formation (EEV production) with increased mouse mortality (pathogenicity). Other vaccinia virus genetic manipulations which affect EEV production inconsistently support this association. However, antisera against vaccinia virus envelope protect mice from lethal challenge, further supporting a critical role for EEV in pathogenicity. Here, we show that the increased comet formation phenotypes of a diverse collection of variola viruses associate with strain phylogeny and geographical origin, but not with increased outbreak-related CFRs; within clades, there may be an association of plaque size with CFR. The mechanisms for variola virus pathogenicity probably involves multiple host and pathogen factors.

Associations between extracellular enveloped virus (EEV) production and pathogenicity have long been hypothesized for orthopoxviruses. Several forms of enveloped orthopoxvirus progeny are antigenically distinct (Appleyard et al., 1971; Smith et al., 2002). Membrane wrapping of intracellular mature virus (IMV) creates intracellular enveloped mature virus (IEV), which is then transported to the cellular membrane via microtubules to form cell-associated enveloped virus (CEV). Release from the cellular membrane yields EEV (Smith et al., 2002). Envelope structure and incorporation of host complement control proteins into the EEV/CEV envelope [potentially facilitating evasion of host complement neutralization (Appleyard et al., 1971; Vanderplasschen et al., 1998)] may facilitate virus dissemination within the host.

In a subset of vaccinia strains, increased EEV production in vitro, as monitored by comet formation, is associated with increased mortality within the mouse intranasal infection model (Payne, 1980). Within this model, passive transfer of antisera against inactivated EEV protects 90% of mice against lethal vaccinia virus infection, while antisera against inactivated IMV does not provide protection (Payne, 1980). These observations support the hypothesis that increased EEV/CEV production results in dissemination of infection within the host and greater pathogenicity. Vaccinia strain Western Reserve (WR), a notable exception, is highly pathogenic yet forms few comets (Payne, 1980). Sequence comparison of four virulence genes demonstrates that WR is phylogenetically distinct from other vaccinia strains (Trindade et al., 2007).

Currently, most studies to evaluate EEV/CEV production and associated virulence use vaccinia virus-WR with genetic modifications in individual envelope proteins. Deletion/alteration of several genes (F12, A33, A36 and B5) causes decreased EEV/CEV production and reduced virulence in mouse infection models (Engelstad & Smith, 1993; Gurt et al., 2006; Parkinson & Smith, 1994; Wolffe et al., 1993; Zhang et al., 2000). However, other mutations within A33, A34 or B5 enhance vaccinia virus-WR EEV/CEV production/release but decrease virulence (Gurt et al., 2006; Katz et al., 2002, 2003; McIntosh & Smith, 1996). Envelope proteins are highly conserved in orthopoxviruses (Aldaz-Carroll et al., 2005; Engelstad & Smith, 1993) and highly recognized by the host immune system. B5 is an immuno-dominant antigen in variola virus-infected humans (Davies et al., 2007) and α-B5 antibodies are primarily responsible for vaccinia virus EEV/CEV neutralization by vaccinia immune globulin (Bell...
et al., 2004; Putz et al., 2006), the recommended treatment for post-vaccination complications (Rotz et al., 2001). The role that variola virus EEV plays in disease pathogenesis is complex and may better be understood holistically within the whole virus context. Here, we evaluated variola virus’s comet and plaque phenotypes in tissue culture, quantified EEV and cell associated virus (CAV) accumulation and assessed their association with virus phylogeny and outbreak-associated case fatality rates (CFRs) as a measure of pathogenicity.

The comet-forming ability of orthopoxviruses, such as vaccinia virus, relates to EEV production (Payne, 1980; Wolfe et al., 1993). Variola isolates \((n=25)\) chosen from diverse geographical regions and years of isolation were evaluated for in vitro comet formation within a liquid overlay and for plaque size within a semi-solid overlay. All variola virus infections were conducted under Biosafety Level 4 conditions, where BSC-40 cell monolayers were infected with each variola strain \([\text{isolation and propagation carried out as described previously (Esposito et al., 2006; Li et al., 2007)}]\). Each virus was diluted in RPMI 1640 medium + 2% fetal bovine serum to achieve ~20–50 p.f.u. per well. After 1 h incubation (35.5 °C, 6% CO₂), the inoculum was removed and monolayers were washed twice with medium. Medium or medium + 1× carboxymethyl-cellulose was added to liquid overlay and semi-solid overlay wells, respectively. After incubation for 4 days, overlays were removed and cells were fixed with 10% formalin in PBS for 20 min. The fix solution was removed and plates were treated with \(4.4 \times 10^4\) Gy gamma irradiation. Comets and plaques were visualized by immunohistochemical staining with polyclonal rabbit α-variola antibody as previously described (Yang et al., 2005).

Visual classification was based upon subjective interpretation of the magnitude of comets formed by each variola strain, from most (Visual Group 1) to least (Visual Group 3) prominent comets (Fig. 1). As a more quantitative measure of comet-forming ability, the percentage of plaques forming

![Figure 1](http://vir.sgmjournals.org) 793

**Fig. 1.** Comet phenotype (upper wells) and plaque size (bottom wells) of 25 variola strains. Strains were classified qualitatively from most (Visual Group 1) to least (Visual Group 3) robust/prominent comets and the percentage of total plaques forming comets are shown on upper wells. The mean maximal plaque sizes (×5.8 magnification) are shown in the corners of the lower wells.
comets was calculated for each variola strain by counting total plaque number (primary plaques of similar size) and number of plaques forming comets (defined as a directional flow of more than four satellite plaques emanating from a primary plaque) per well within the photograph ($\times 3.9$ magnification). The mean count reflected three different individuals’ counts of duplicate wells; all plaques within 1 cm of the well boundary on the photographs were excluded due to potential interference in comet formation. Variola isolates demonstrated diverse comet-formation phenotypes, which were reproducibly seen for each isolate in duplicate wells. Although the variation in comet-forming plaques could be due to microheterogeneity within each isolate, there were no majority ‘subpopulations’ identified during sequencing (Esposito et al., 2006). Variola isolates with nearly identical nucleotide sequences (Esposito et al., 2006) displayed similar comet phenotypes [Figs 1 and 2, e.g. ETH72_16 (Group 2, 93 %) and ETH72_17 (Group 2, 89 %)]. Reproducibility of comet quantification was within 20 % coefficient of variation (based on mean and sd of counts) for any given isolate except UNK46_harv and SOM77_alni, which had 67 and 21 % of plaques forming comets, respectively, grouping them with the lowest comet-producing strains.

In vitro comet phenotype was compared to geographical distribution, phylogeny (Li et al., 2007) and reported CFRs

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Fig. 2. Maximum-likelihood phylogram of 47 variola virus strains (Li et al., 2007), including geographical location of isolation (number of isolates given in parentheses), reported CFRs (Esposito et al., 2006) and plaque/comet phenotype.
Relating variola plaque phenotype to phylogeny

Subjective visual classifications and quantification of comet-forming ability segregated variola virus strains in a manner correlating with phylogeny and geographical origin (Fig. 2). Non-parametric analysis (Mann–Whitney exact two-tailed t-test) of the mean proportion of comet-forming plaques between variola virus primary clades demonstrated a significant difference (P=0.0002, SAS Version 9.1) between clade I (n=18) and clade II (n=7) [80.19 ± 17.95 % (SD) and 96.67 ± 2.93 % (SD), respectively], supporting the hypothesis that there is a phenotypic trait that complements genomic differences (Figs 1 and 2). Increased comet production was seen with primary clade II isolates [from West Africa and Brazil (Alastrim minor)]. Of the seven primary clade II strains, almost all were categorized by both methods as the highest comet producers (Fig. 2), six had >95 % plaques producing comets. The one exception, BRZ66_gar (92 %), was a laboratory-derived strain with an unknown number of passages through tissue culture cells; laboratory manipulations could possibly have caused adaptations that might explain some phenotypic differences compared with other primary clade II strains. Within primary clade I, only two strains [IND64_vel4 (Group 1, 96 %) and BSH74_sol (Group 2, 95 %)] of 18 displayed ≥95 % comet production and only four strains displayed between 90 and 95 % production. The independent and reproducible categorization of the vast majority of primary clade II strains as the highest comet producers, and the lack of prominent comet phenotypes within primary clade I, increases confidence in the relationship of higher EEV production/release with variola virus primary clade II. The Alastrim isolates within this clade, historically considered to be the least pathogenic strains as demonstrated by clinical outcome, have reported CFRs <1 %. Highly pathogenic Asian and Oriental variola isolates (e.g. NEP73_175, KOR47ilee, IND64_vel4 and BSH74_sol) did not uniformly express EEV, as measured by comet production (Figs 1 and 2). Thus, increased variola virus EEV production alone does not relate to increased mortality (CFR).

For plaque size analysis, three of the largest primary plaques (of similar size with uniform circular shape) for each isolate were measured from random locations within each of the photographs of duplicate wells (×5.8 magnification). The measured plaques were >1 cm from the well boundary on the photographs to ensure there was no interference in plaque formation. The mean plaque size (in mm; ×5.8 magnification) for each isolate was determined from six plaque measurements. Consistent with observations from vacinia strains where increased EEV production/release does not relate to increased plaque size (Blasco & Moss, 1992; Katz et al., 2002; Payne, 1980), clade II variola virus strains with robust comets displayed an equivalent maximal plaque size as clade I isolates (6.7–9.2 mm and 5.3–9.7 mm, respectively, at ×5.8 magnification) (Figs 1 and 2). However, within clades, mean plaque size was significantly larger for clade I isolates from Asia, Bangladesh, the Orient or the Middle East (n=8) (mean ± SD, 8.46 ± 0.92 mm) versus the lower CFR of non-West-African isolates (n=8) (mean ± SD, 7.56 ± 1.07 mm) (P-value ≤0.0001, two-tailed t-test). Similarly, clade II isolates from West Africa (n=4) (mean ± SD, 8.54 ± 0.59 mm) demonstrated significantly larger plaque size and higher CFRs than Brazil/Alastrim isolates (n=3) (mean ± SD, 7.06 ± 0.64 mm) (P-value ≤0.0001, two-tailed t-test). Therefore, an increase in the mean plaque size of variola virus was not associated with increased EEV (comet) production but, within primary clades, did relate to increased CFRs.

The prominent comet phenotype for primary clade II isolates implied that these ‘less-virulent’ strains produce/release more EEV. To test this hypothesis, growth kinetic assays were performed to quantify CAV and EEV production by variola strains from the three comet formation groups that were isolated from diverse geographical locations and in a range of years. These were high comet-producers isolated in Brazil in 1966 [BRZ66_39] and Sierra Leone in 1968 [SLN68_258]; intermediate comet-producers isolated in Bangladesh in 1974 [BSH74_sol] and Sudan in 1947 [SUD47_jub]; low comet-producers isolated in Nepal in 1973 [NEP73_175] and Somalia in 1977 [SOM77_ali]. Cell monolayers (BSC-40) were infected with each variola strain as described above, at ~5 p.f.u. per cell. After adsorption for 1 h (35.5 °C, 6 % CO₂), the inoculum was removed and monolayers were washed twice before addition of growth media. Supernatants containing released EEV were collected at each time point [2, 6, 12, 18, 24, 36, 48 and 72 h post-infection (p.i.)] and titres on E6 cells were determined as previously described (Yang et al., 2005), in the presence of antibody (J2D5, 1:1000 dilution) to neutralize any contaminating IMV. Media was added to each of the duplicate wells to harvest CAV by scraping monolayers into the media and freezing the lysate at −70 °C. Titres for all CAV were determined at the same time after freeze-thawing the lysate and sonicating on ice for 1.5 min. To titrate, inoculum was serially diluted in media and plated onto confluent E-6 cell monolayers. After 1 h incubation (35.5 °C, 6 % CO₂), inoculum was removed and medium was added to each well. Incubation continued for 4 days until the cells were stained with 2 × crystal violet.

Accumulation of CAV (Fig. 3a) was higher in strains NEP73_175, SOM77_ali and SUD47_jub (~2–3 log increase) than in strains BSH74_sol, BRZ66_39 and SLN68_258 (~1–1.5 log increase). The number of released EEV was maximal for all strains at ~18–24 h p.i. (Fig. 3b). The highest levels of EEV were produced by BRZ66_39 and SLN68_258, despite these strains producing <2 log increase in CAV (Fig. 3a and b). The highest EEV production correlated with clade II variola virus strains; differences in EEV production between intermediate and low comet producers were more difficult to discern during analysis of growth kinetics. Evaluation of the ratio of EEV to CAV at maximal EEV release (24 h p.i.) confirmed that...
EEV production is directly related to the qualitative comet formation ability of variola virus (Fig. 3c). Higher EEV production/release and comet formation is associated with the less pathogenic variola virus primary clade II. Relatively small alterations in EEV/CEV envelope proteins influence vaccinia virus pathogenicity with variable effects on immunogenicity within animal models (Gurt et al., 2006; Katz et al., 2003). A single amino acid (aa) substitution within B5 or a 35 aa truncation of A33 dramatically increases EEV production \textit{in vitro} but reduces virulence upon mouse intranasal infection (Katz et al., 2003). We compared variola virus sequences of each homologue to vaccinia virus-characterized EEV and IEV predicted proteins. Analysis of 47 variola virus sequences (Esposito et al., 2006) demonstrated that both B5 and A33 aa sequences were conserved between primary clades, suggesting that EEV production differences do not result from genetic alterations in these EEV/CEV proteins. Further analysis of homologues to IEV and EEV envelope proteins (A34, A36, A56, F12 and F13) showed clade-specific differences only within A56 (haemagglutinin) and F12 (see Supplementary Fig. S1, available in JGV Online). No conserved aa changes that related to plaque size differences seen between subclades within variola virus clade I were found within these homologues, but four conserved aa changes within F12 differentiated variola virus clade II subclades, perhaps relating to increased plaque size of the West African isolates. The F12 protein is associated with IEV and is required for egress to the cell surface (Herrero-Martínez et al., 2005; van Eijl et al., 2002). In the absence of F12, the integrity of vaccinia virus IMV and IEV are preserved, but the virus produces small plaques, decreased CEV (>99%) and EEV (sevenfold less) and diminished virulence (Herrero-Martínez et al., 2005; Zhang et al., 2000). Although haemagglutinin is not necessary for normal EEV production (Sanderson et al., 1998), mutations in the C-terminal region affect kinetics of IMV trafficking through the envelope formation process (Shida, 1986). Potentially, the few clade-specific and subclade-specific aa alterations within F12 or the insertion and point mutation in the near-transmembrane region of haemagglutinin may be associated with increased EEV production/release by primary clade II variola virus isolates and increased plaque size of West African isolates (Supplementary Fig. S1).

Previous research has focused upon the effect that single gene mutations have upon vaccinia virus EEV production. Our study assessed differences in variola virus comet phenotypes, EEV production, plaque morphology and their relation to phylogeny and outbreak CFRs as a measure of pathogenicity. Clade II isolates, historically considered ‘less virulent’, were classified by two independent methods as the highest EEV and comet-producing strains. EEV production alone does not correlate with pathogenicity; other viral and host factors, such as the host immune and inflammatory response (Stanford et al., 2007), are likely to be related to variola virus pathogenicity and subsequently CFRs. A virus-related, biologically beneficial consequence of increased EEV production/release by clade II variola virus isolates, which have decreased virulence, may be to promote virus dissemination between human hosts. A role of EEV in virus transmission was suggested through observations of vaccinia

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**Fig. 3.** The CAV (a) and EEV (b) titres of six variola strains were calculated at different times p.i. Data shown are means ± SD of data from duplicate wells. (c) EEV/CAV ratio at the time of maximal EEV release (24 h p.i.).
virus strain IHDJ intranasal mouse infection; EEV and CEV accumulate in nasal epithelia 5 days p.i. (Payne, 1980; Payne & Kristensson, 1985). It may be that increased EEV production provided for efficient respiratory spread between hosts but also diminished variola virus pathogenicity due to EEV/CEV-specific host immune recognition and clearance.

The lack of a temporal phylogenetic relationship and presence of a geographical phylogenetic relationship within this collection of variola isolates argue against a pandemic sweep of disease (Li et al., 2007). The strong relationship between variola strain phylogeny, geographical origin and in vitro phenotype (comet-formation ability, EEV production/release and plaque size) could represent regional geographical evolution of variola virus with its human host. The biological properties associated with smallpox human pathogenesis are not completely known; further characterization of in vitro phenotypes of variola virus clades may lead to identification of those factors involved in pathogenicity and subsequently to novel targets for antiviral therapies. Finally, the relationship between comet formation (EEV production) and immunogenic protection or potential involvement in transmission should be explored further.

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