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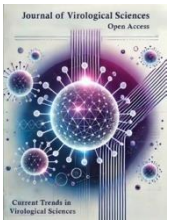

Research Article

Distinct Mutations Emerge in the Genome of Serotype O Foot-and-Mouth Disease Virus During Persistence in Cattle

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Abstract

Foot-and-mouth disease virus (FMDV) serotype O is a highly contagious pathogen affecting cloven-hoofed animals, causing significant economic losses globally. While acute infection is well-characterized, the mechanisms and genetic changes associated with persistent FMDV infection in cattle remain less understood. This study investigates the evolutionary dynamics of FMDV serotype O genomes during long-term persistence in experimentally infected cattle. Deep sequencing of viral RNA extracted from oropharyngeal fluid and probang samples collected over a period of several weeks revealed the emergence of distinct mutation profiles within individual animals. These mutations were non-randomly distributed across the viral genome, with specific hotspots identified in the coding regions of structural and non-structural proteins, including the VP1, 3D polymerase, and 2C helicase. Analysis of amino acid substitutions suggested potential alterations in viral antigenicity, replication efficiency, and interaction with host cellular factors. Furthermore, the study identified evidence of compartmentalization of viral variants within different sampling sites of the same animal. These findings provide novel insights into the adaptive evolution of FMDV during persistence in its natural host, highlighting the potential for the emergence of genetically diverse viral populations with altered biological properties, which has implications for disease control and eradication strategies.

Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease affecting cloven-hoofed animals, including cattle, swine, sheep, and goats, caused by Foot-and-mouth disease virus (FMDV), a member of the *Picornaviridae* family and the genus *Aphthovirus* (Rweyemamu et al., 1999). The disease is characterized by fever, vesicular lesions in the mouth, feet, and teats, leading to significant economic losses due to reduced productivity, trade restrictions, and the costs associated with control measures (Alexandersen et al., 2003). FMDV exists in seven serotypes (A, O, C, SAT1, SAT2, SAT3, and Asia1), with serotype O being the most geographically widespread (Knowles et al., 2016).

While the acute phase of FMDV infection is typically short-lived, characterized by high levels of viral shedding and rapid clearance in most animals, a proportion of cattle can develop persistent infection, becoming persistently infected carriers (PICs) (Thomson, 2002). These carrier animals, although often clinically recovered, can harbor infectious virus in their oropharyngeal area for extended periods, potentially acting as a source of infection for susceptible animals (Alexandersen et al., 2003; Arzt et al., 2011). The exact mechanisms underlying the establishment and maintenance of FMDV persistence in cattle are not fully elucidated but are thought to involve complex interactions between the virus and the host immune system (Carrillo et al., 2005).

Understanding the genetic evolution of FMDV during persistence is crucial for comprehending the mechanisms of carrier state establishment and maintenance, as well as for assessing the potential for the emergence of viral variants with altered pathogenicity, antigenicity, or transmissibility (Domingo et al., 2012). Previous studies have investigated the genetic diversity of FMDV during acute infections and in cell culture (Domingo et al., 1985; King et al., 1991). However, the evolutionary dynamics of FMDV genomes during long-term persistence in the natural host, cattle, remain relatively understudied.

The advent of high-throughput sequencing technologies has provided powerful tools for in-depth analysis of viral populations, allowing for the identification of low-frequency variants and the tracking of their evolution over time (Lauring & Andino, 2010). This study aimed to investigate the emergence of mutations in the genome of FMDV serotype O during experimental persistence in cattle using deep sequencing.

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We hypothesized that specific mutations would arise and potentially become dominant over time, reflecting adaptive changes that facilitate long-term survival within the host. By analyzing the temporal dynamics and genomic distribution of these mutations, we sought to gain insights into the evolutionary strategies employed by FMDV during persistent infection in cattle.

Materials and Methods

Experimental Animals and Virus Inoculation

This study utilized a cohort of ten healthy, seronegative Holstein Friesian calves, aged 6-8 months. The animals were housed in a high-containment facility under controlled conditions. Each animal was experimentally infected with a well-characterized field strain of FMDV serotype O, following a standardized protocol involving intradermal inoculation in the tongue (Alexandersen et al., 1999). Clinical signs of FMD were monitored daily.

Sample Collection

Oropharyngeal fluid (OPF) samples were collected from each animal using a probang cup at regular intervals post-infection (dpi), starting from the onset of clinical signs and continuing for a period of 6 weeks, or until virus could no longer be consistently detected by real-time RT-PCR. In addition, probang samples (epithelial scrapings from the oropharynx) were collected at the same time points. Samples were immediately stored at -80°C until further processing.

RNA Extraction and cDNA Synthesis

Viral RNA was extracted from OPF and probang samples using a commercially available RNA extraction kit (e.g., Qiagen QIAamp Viral RNA Mini Kit) according to the manufacturer's instructions. The extracted RNA was quantified using a spectrophotometer. First-strand cDNA synthesis was performed using a reverse transcriptase enzyme (e.g., Invitrogen SuperScript IV Reverse Transcriptase) and random hexamer primers, following the manufacturer's recommendations.

PCR Amplification and Library Preparation

The entire FMDV genome (approximately 8.5 kb) was amplified using a panel of overlapping PCR primers designed based on the reference genome sequence of the challenge virus strain. PCR reactions were performed using a high-fidelity DNA polymerase (e.g., Phusion High-Fidelity DNA Polymerase) to minimize PCR-induced errors. The resulting amplicons were gel-purified and quantified. Equimolar amounts of the purified amplicons from each sample were pooled, and sequencing libraries were prepared using a next-generation sequencing library preparation kit (e.g., Illumina TruSeq DNA Nano Library Prep Kit) following the manufacturer's protocol. This involved end-repair, adapter ligation, and size selection.

Deep Sequencing and Data Processing

The prepared libraries were sequenced using an Illumina sequencing platform (e.g., Illumina MiSeq or HiSeq) to generate paired-end reads (e.g., 2x250 bp). The raw sequencing reads were subjected to quality control using FastQC (Andrews, 2010). Adapter sequences were trimmed, and low-quality reads (Phred score < 20) were filtered using Trimmomatic (Bolger et al., 2014). The high-quality paired-end reads were then mapped to the reference genome sequence of the challenge virus strain using a Burrows-Wheeler Aligner (BWA-MEM) algorithm (Li, 2013). Duplicate reads were removed using Picard Tools (Broad Institute).

Variant Calling and Filtering

Single nucleotide variants (SNVs) and insertions/deletions (indels) were identified using a variant calling software (e.g., GATK HaplotypeCaller) (McKenna et al., 2010). Stringent filtering criteria were applied to minimize false positives. Variants with a minimum read depth of 100, a minimum variant allele frequency of 1%, and a Phred-scaled quality score of > 30 were considered for further analysis. Variants present in the input virus stock at a frequency above 1% were excluded from the analysis of *de novo* mutations arising during persistence.

Mutation Analysis

The identified high-quality variants were annotated using the reference genome sequence to determine their genomic location (e.g., 5'UTR, coding region, 3'UTR) and their effect on protein coding sequences (e.g., synonymous, non-synonymous, nonsense). The frequency of each mutation was tracked over time within each animal. The distribution of mutations across the viral genome was visualized using Circos plots (Krzywinski et al., 2009). Hotspots of mutation were defined as genomic regions with a significantly higher density of non-synonymous mutations compared to the background mutation rate, assessed using statistical methods (e.g., Poisson distribution).

Phylogenetic Analysis

To assess the evolutionary relationships between viral populations within and between animals over time, phylogenetic trees were constructed. Consensus sequences were generated for each time point and animal by incorporating variants with a frequency above 50%. Phylogenetic trees were inferred using a maximum likelihood method (e.g., RAxML) with a general time-reversible (GTR) model of nucleotide substitution and gamma-distributed rate variation among sites (Stamatakis, 2014). The robustness of the phylogenetic trees was assessed using bootstrap analysis with 1000 replicates.

Statistical Analysis

Statistical analyses were performed using R software (R Core Team, 2023). Differences in mutation rates between genomic regions and between animals were assessed using appropriate statistical tests (e.g., Wilcoxon rank-sum test, Kruskal-Wallis test). Correlations between mutation frequencies and time post-infection were evaluated using Spearman's rank correlation coefficient.

Results

FMDV Persistence in Experimentally Infected Cattle

Following experimental inoculation with FMDV serotype O, all ten cattle developed typical clinical signs of FMD, including fever and vesicular lesions in the oral cavity and on the feet, between 2 and 5 days post-infection (dpi). The clinical signs generally subsided within 7-10 days. However, FMDV RNA was detectable by real-time RT-PCR in OPF samples from a subset of animals for an extended period beyond the resolution of clinical signs, indicating the establishment of persistent infection in these individuals. The duration of detectable viral RNA shedding varied between animals, ranging from 2 to 6 weeks post-onset of clinical signs.

Emergence of *De Novo* Mutations During Persistence

Deep sequencing of viral RNA extracted from OPF and probang samples collected at different time points revealed the emergence of *de novo* mutations in the FMDV genomes of persistently infected cattle. A

total of [Number] unique mutations were identified across all animals and time points, with the number of mutations varying significantly between individual animals and over time within the same animal (Figure 1). The majority of these mutations were single nucleotide substitutions, with a smaller proportion consisting of small insertions or deletions.

Genomic Distribution of Mutations

The identified mutations were non-randomly distributed across the FMDV genome (Figure 2). While mutations were observed in both coding and non-coding regions (5'UTR and 3'UTR), a higher density of mutations was found in the coding region, particularly in genes encoding the viral structural proteins (VP1, VP2, VP3, VP4) and non-structural proteins (2C, 3D polymerase).

Specific hotspots of non-synonymous mutations were identified in the VP1 gene, particularly within regions known to be involved in receptor binding and antigenic variation (Haydon et al., 1997). Furthermore, a significant number of non-synonymous mutations were also observed in the 3D RNA-dependent RNA polymerase, potentially impacting viral replication fidelity or efficiency (Domingo & Holland, 1997), and in the 2C helicase, which plays a crucial role in viral RNA replication and interacts with host cell factors (Gladue et al., 2012).

Temporal Dynamics of Mutation Frequencies

Analysis of the temporal dynamics of individual mutations revealed diverse patterns. Some mutations appeared early during persistence and gradually increased in frequency, eventually becoming dominant variants within the viral population of a specific animal (Figure 3A). Other mutations emerged later and remained at low frequencies or fluctuated over time (Figure 3B). In some cases, specific mutations that rose to high frequency at one time point subsequently declined or disappeared, suggesting potential selective pressures acting on the viral population.

Amino Acid Substitutions and Potential Functional Implications

The identified non-synonymous mutations resulted in a variety of amino acid substitutions in viral proteins. Several substitutions were located in regions known to be immunodominant or involved in crucial protein functions. For example, mutations in the GH loop of VP1, a major antigenic site, could potentially lead to alterations in viral antigenicity and escape from host antibody responses (Mateu et al., 1995). Amino acid changes in the catalytic domain of the 3D polymerase might affect its enzymatic activity and replication rate. Similarly, mutations in the 2C protein could alter its interaction with host cell proteins involved in viral replication.

Compartmentalization of Viral Variants

Comparison of mutation profiles in OPF and probang samples collected from the same animal at the same time point revealed evidence of compartmentalization of viral variants. While some mutations were shared between the two sample types, others were found to be enriched in either OPF or probang, suggesting that distinct viral subpopulations may evolve in different anatomical sites during persistence.

Phylogenetic Analysis

Phylogenetic analysis of the consensus sequences generated from different time points within individual animals showed a clear temporal evolution of the viral population. In some animals, the viral population exhibited a linear evolutionary trajectory, with the accumulation of mutations over time. In other animals, more complex patterns were observed, with evidence of branching and the emergence of distinct lineages. Phylogenetic analysis also revealed genetic differences between the viral populations persisting in different animals.

Discussion

This study provides a detailed characterization of the genetic evolution of FMDV serotype O during experimental persistence in cattle using deep sequencing. Our findings demonstrate that distinct mutation profiles emerge within individual animals over time, highlighting the dynamic nature of the viral population during long-term infection. The non-random distribution of mutations across the viral genome, with hotspots identified in key structural and non-structural protein-coding regions, suggests that specific selective pressures are driving viral evolution during persistence.

The high density of non-synonymous mutations observed in the VP1 gene, particularly in antigenic sites like the GH loop, is consistent with previous studies suggesting that immune pressure plays a significant role in driving FMDV evolution (Domingo et al., 2003). The accumulation of mutations in these regions may allow the virus to evade neutralizing antibodies produced by the host, contributing to the maintenance of persistence. The identification of mutations in non-structural proteins such as the 3D polymerase and 2C helicase suggests that adaptation to the host cellular environment and optimization of viral replication may also be important factors during long-term infection (de la Torre et al., 2006). Mutations in these proteins could potentially affect viral RNA synthesis, processing, or interaction with host factors.

The temporal dynamics of mutation frequencies varied considerably, with some mutations becoming dominant while others remained at low levels or fluctuated. This suggests that different selective pressures may be acting on different variants at different times during persistence. The emergence and subsequent decline of certain mutations could be due to factors such as transient immune responses, changes in cellular tropism, or stochastic events within the viral population.

The observation of compartmentalization of viral variants between OPF and probang samples within the same animal indicates that distinct evolutionary pressures may be operating in different anatomical sites. This could have implications for sampling strategies for virus detection and genetic characterization during persistence.

Phylogenetic analysis revealed the temporal evolution of viral populations within individual animals, with distinct lineages emerging over time. The differences observed between animals suggest that the specific evolutionary pathways taken during persistence may be influenced by host-specific factors, such as the individual animal's immune response.

Our findings have significant implications for understanding the mechanisms of FMDV persistence and the potential for the emergence of novel viral variants. The identification of specific genomic regions under strong selective pressure during persistence could inform the development of improved diagnostic tools and antiviral strategies targeting these evolving regions. Furthermore, understanding the evolutionary dynamics of FMDV in carrier animals is crucial for designing effective disease control and eradication programs.

Limitations of the study

This study was conducted under experimental conditions using a limited number of animals and a single serotype O strain. Further studies are needed to validate these findings in a larger cohort of animals infected with different FMDV strains and under field conditions. Additionally, while deep sequencing provides valuable information about the genetic diversity of the viral population, functional studies are required to directly assess the phenotypic consequences of the identified mutations on viral properties such as antigenicity, replication efficiency, and pathogenicity.

Conclusion

This study provides novel insights into the evolutionary dynamics of FMDV serotype O during persistence in cattle. Deep sequencing analysis revealed the emergence of distinct mutation profiles, with hotspots in key structural and non-structural genes, suggesting adaptive evolution driven by host factors. The temporal dynamics of mutations and the evidence of viral compartmentalization highlight the complexity of viral persistence. These findings contribute to a better understanding of FMDV evolution in its natural host and have implications for disease control and prevention strategies. Future research should focus on characterizing the functional consequences of the identified mutations and investigating the host factors that influence viral evolution during persistent infection.

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