Maternal Transmission is the Major Mode of Ovine Lentivirus Transmission in a Ewe Flock: A Molecular Epidemiology Study

By

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To the Faculty of Washington State University:

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Abstract
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Transmission of ovine progressive pneumonia virus (OPPV), a lentivirus of sheep, occurs through both maternal and non-maternal means. Currently, the contribution of each route to the overall flock OPPV prevalence is poorly understood since previous serological epidemiologic studies lacked the ability to accurately track routes of transmission within an infected flock. In this study, the amount of maternal OPP transmission was assessed in a naturally infected ewe flock by applying molecular analyses to proviral sequences derived from peripheral blood leukocytes of OPP positive dam-daughter pairs (N=40). Both proviral envelope (*env*) and long terminal repeat (*ltr*) sequences, separately and combined, were utilized in the following two sequence analysis methods: phylogenetic analysis and pairwise distance calculations. True maternal transmission events were defined as agreement in 2 out of the 2 sequence analysis methods. Using this criterion, proviral *env* sequences resulted in a 14.3% maternal transmission frequency, and proviral *ltr* sequences resulted in a 10% maternal transmission frequency. Both proportions of maternal transmission varied significantly from equality ($P < 0.0001$). This indicates that the remaining 85.7-90% of daughters are infected via non-maternal transmission. This is also the first study to calculate the OPP proviral rate of change for *env* and *ltr* genes.

Accurately defining the routes of OPPV transmission provides critical epidemiological data supporting management intended to reduce flock transmission and viral dose.

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Dedication

This thesis is dedicated to my loving wife Brie who has motivated and enabled me to excel in my research and work.

CHAPTER ONE

INTRODUCTION

Ovine progressive pneumonia virus (OPPV) is a lentiviral infection of sheep in the United States, and is part of a family of retroviruses of the genus lentiviridae that also includes human immunodeficiency virus (HIV), equine infectious anemia virus (EIAV), caprine arthritis-encephalitis virus (CAEV), maedi-visna virus (MVV), and feline immunodeficiency virus (FIV). A range of OPPV lesion severity is found in the lungs, mammary glands, joints and/or the brains of infected animals where the animals may or may not display clinical signs such as dypsnea, mastitis, arthritis, cachexia and ataxia. In terms of the small ruminant lentiviruses (SRLV), OPPV is more closely related to caprine arthritis encephalitis virus (CAEV) than maedi visna virus based on the envelope (*env*) gene (Herrmann et al., 2004). OPPV can be experimentally transmitted to goats, and CAEV can be experimentally transmitted to sheep (Banks et al, 1983). In addition, several phylogenetic studies on different SRLV genes suggest that there is natural transmission of the sheep viruses (OPPV and MVV) to goats and natural transmission of the goat virus (CAEV) to sheep (Zanoni et al., 1998; Chebloune et al., 1996; Rolland et al., 2002; Pisoni et al., 2005 & 2007; Reina et al., 2006; Shah, 2004a&b). These experimental and natural transmission studies suggest that OPPV, MVV, and CAEV have no small ruminant host specific requirements for successful transmission.

Currently, transmission of OPPV/MVV in sheep is believed to occur maternally and non-maternally; however, the contribution of each to overall transmission remains unknown. Maternal transmission is defined as transmission of OPP between dam and progeny at any point during their lives, regardless of route. Conversely, non-maternal transmission is defined as transmission between any animals that are not dam-progeny sets, regardless of route.

Maternal and non-maternal transmission through respiratory secretions is believed to be a source of MVV transmission between animals (Sigurdsson et al., 1953; DeBoer et al., 1979; Houwers et al., 1983; Alvarez et al., 2006; McNeilly et al., 2008). This is primarily based upon the fact that both cell free virus and cell associated virus are found in the bronchial alveolar fluid of infected sheep (Lujan et al., 1994; Brodie et al., 1995; McNeilly et al., 2008). In addition, since cell free and cell-associated MVV/OPPV have been detected in colostrum/milk of sheep, and cell free CAEV has been detected in colostrum/milk of goats, maternal transmission from dam to progeny and non-maternal transmission through aerosolization of milk in dairy operations are also thought to contribute to transmission (Cutlip et al., 1981; Adams et al., 1983; East et al., 1987; Lerondelle et al., 1990; Herrmann-Hoesing et al., 2007a). The detection of OPP virus concurrently with *Brucella ovis* infection and OPP proviral DNA detected in ram semen suggests that OPPV has the potential to transmit via sexual transmission (ram to ewes) or paternal transmission (sire to progeny) (de la Concha-Bermejillo et al., 1996; Peterson et al., 2008). Besides these transmission routes and sources, there could be iatrogenic and man-made forms of non-maternal transmission through the re-use of needles for vaccination, shearing equipment, tail-docking, and castration devices; however, there has been no experimental evidence proving that any of these sources are significant to overall transmission. The role of sheep keds (*Melophagus ovinus*) and sheep lungworms (*Muellerius capillaris*) in the transmission of OPPV has been previously investigated but no connection with transmission of OPPV could be established (Siggurdsson et al., 1953; De Boer et al., 1979).

Maternal transmission (dam to progeny) includes the antepartum (prior to birth) or *in utero*, intrapartum (during birth), and postpartum periods. Evaluating lung and lymph node tissues from 1) cesarian derived lambs showed that *in utero* transmission occurs 5.9% of the

time, and 2) neonates receiving no colostrum post-parturition showed that *in utero* and intrapartum transmission occurs 5.7% of the time (Cutlip et al., 1981). The mechanism for *in utero* transmission is unknown but could involve cells at the maternal-fetal interface or maternal blood cells transferring to the fetus. Until recently, most transmission was thought to occur maternally through postpartum contact and colostrum/milk. This assumption was made based upon previous studies in which lambs were removed from their MVV seropositive dam immediately after birth and placed into a negative flock, so that there is no chance for either lactogenic transmission or contact transmission. Under these conditions, 100% of the lambs remained seronegative for the duration of their lives (De Boer et al., 1979). Furthermore, this led to epidemiological studies tracking OPPV/MVV seroprevalence in the context of maternal lines in flocks, and if both a dam and a lamb were seropositive, it was assumed that the infection was transmitted from dam to lamb (Houwers et al., 1989; Brodie et al., 1994). However, unless the dam and lamb are removed from the rest of the infected flock, non-maternal transmission may have contributed to dam to lamb transmission. Current combined studies indicate that postpartum maternal transmission through contact and colostrum/milk prior to weaning (separation of dam and progeny) is inefficient (Alvarez et al., 2005; Herrmann-Hoesing et al., 2007a), and this inefficiency may be partially due to the concurrent presence of maternally transferred antibody in the colostrum/milk with virus (Herrmann-Hoesing et al., 2007a).

Currently, there are no data delineating the efficiency of maternal transmission versus non-maternal transmission that occurs in an endemic SRLV flock. With molecular methods available to sequence viral genes in individual ewes, transmission events can be identified as maternal transmission and non-maternal transmission based upon viral gene sequences and phylogenetic analysis. The use of viral sequence in the phylogenetic analysis of viral genes has

been used extensively in HIV epidemiological research. The rapid rate at which mutations accumulate in the HIV genome through the low fidelity of reverse transcriptase has been used to track transmission events in many geographical regions (Bao et al., 2008; Brendell et al., 2003; Leitner et al., 1996). A classic example of this is the case of the HIV positive Florida dentist and the subsequent use of viral fingerprinting to determine the origin of the infection (Ou et al., 1992).

Our previous study showed that there was transmission of OPPV through contact and colostrum/milk to 22 lambs using the detection of provirus through week 22 of age; however, at the end of 4 years, one unrelated co-mingled naive ewe and none of the 22 lambs became persistently infected with OPPV (Herrmann-Hoesing et al., 2007a). However, at the end of 6 years, one of the 22 lambs seroconverted and became persistently antibody and proviral positive. In the present study, envelope (*env*) sequences were evaluated in the dams and progeny from our previous study to determine the source of OPPV transmission. Furthermore, *env* and long terminal repeat (*ltr*) sequences were evaluated in dam-daughter pairs in an Idaho flock endemic with OPPV to evaluate whether daughters acquired OPPV from their dams or from some other non-maternal source.

CHAPTER TWO

MATERIALS and METHODS

Animals

In February 2002, ten gravid 6-year-old ewes of Rambouillet, Columbia and Polypay breeds originating from the U.S. Sheep Experiment Station in Dubois, ID were selected based upon the presence of anti-OPPV antibodies in their serum and transported to Pullman, WA. OPP provirus was also demonstrated in the peripheral blood leukocytes of these 10 serologically positive sheep. The ten pregnant ewes gave birth to 23 lambs in April 2002. The lambs were numbered LMH 21-43. Lambs were allowed to naturally suckle colostrum/milk from and maintain contact with the dams for 32 weeks. After this point, the lambs were separated from their dams but still had nose contact through a fence. In March 2003, one unrelated, OPPV negative animal (112-45) was placed with the positive dams until the dams were euthanized in April 2005. After this point, the unrelated animal was placed in with the lambs.

Three hundred forty two dam-daughter pairs consisting of 175 Polypay, 70 Columbia, 97 Rambouillet pairs were identified in an ewe flock blood sampling of 1,098 sheep from the U.S. Sheep Experiment Station in Dubois, ID. Of these dam-daughter pairs, 40 dam-daughter pairs consisting of 25 Polypay, 9 Columbia, and 6 Rambouillet pairs were serologically and peripheral provirus positive for OPPV. The maximum age difference between dam and daughter pairs was 3 years. Anti-OPPV antibodies were measured using serum and a validated competitive ELISA, and provirus levels were measured using a validated OPPV qPCR (Herrmann et al., 2003; Herrmann-Hoesing et al., 2007b).

Blood isolation/DNA isolation

The animals were manually restrained and bled by jugular venous puncture into 10 ml Vacutainer tubes containing 10 mM EDTA (for peripheral blood leukocyte isolation) and 10ml Vacutainer tube containing no anti-coagulant. All animal handling procedures were performed in compliance with standards set by the institutional animal care and use committee of [insert group here]. Serum and peripheral blood leukocytes were isolated from 10 ml of whole blood as previously described (Herrmann-Hoesing, 2007b). DNA isolations from PBLs were performed by following the manufacturer's directions for 10 million cells using the Puregene cell kit (Qiagen)

Amplification of viral genes

OPPV *env* and *ltr* genes were amplified using two rounds of PCR with sequence specific oligonucleotide primers (See Table 1). The PCR amplification was performed on 100-500ng of DNA using 1x PCR dilution buffer, 2.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM forward and reverse primer, and 0.02 U/μl of Taq polymerase purchased from Fisher Scientific. The PCR parameters were as follows for both *ltr* and *env* amplification: one cycle of 5 minutes at 95°C; 35 cycles of 30 seconds at 95°C, 30 seconds at 59°C and 1 minute 30 seconds at 72°C followed by one cycle of 72°C for 7 minutes. PCR amplification produced a ~1,600 bp envelope fragment and a ~670 bp ltr fragment.

Cloning, plasmid isolation, and sequencing

Cloning and plasmid isolation procedures were performed as previously described (Herrmann et al., 2004). Positive clones were sequenced at the Washington State University bioinformatics

core facility on an ABI Prism 3730 DNA sequencer using standard di-terminator methods with M13 and sequence specific oligonucleotide primers (See Table 1). The average number of *env* and *ltr* proviral sequences generated per animal was 4 and 8, respectively.

Nucleotide sequence accession numbers

The GenBank accession numbers for *ltr* sequences used in this study are HM052841 – HM053433 and HM060336-HM060528. The GenBank accession numbers for *env* sequences used in this study are HM056780-HM057098, HM060401-HM060528, and HM067709-HM067742.

Programs for consensus sequence generation and alignment

Sequence alignments were performed using the DNAstar Lasergene v. 7.1 software package and further refined using the Se-Al program to ensure all *env* sequences were in coding frame (http://tree.bio.ed.ac.uk/software/seal/). Consensus sequences were generated using the DNAstar Lasergene seqman and seqbuilder programs.

Phylogenetic analyses

Phylogenetic analyses using maximum likelihood and Bayesian methods were performed using consensus sequences. Parameters important for correct statistical model selection for the phylogenetic analyses were estimated using the MrModeltest v2.3 (Nylander et al., 2004) command block executed in PAUP*4.0b10 (Swofford et al., 2003) (Table 2). Both phylogenetic analyses were performed using the general time reversible model with a measure of rate heterogeneity and invariant sites. Maximum likelihood analysis was performed as executed in

the PhyML program (Guindon et al., 2003), and Bayesian analysis was performed using MrBayes v3.1 (Ronquist et al., 2002).

Pairwise divergence

Pairwise divergence calculations were performed on all sequences using the same parameter estimates as the phylogenetic analyses and executed in PAUP*4.0b10. Pairwise divergence values between dam and daughter were compared against pairwise divergence values between dam and unrelated ewes using a Wilcoxon rank sum test and Bonferroni correction to control type I error rates (SAS v. 9.1, SAS Institute, Cary NC).

Evolutionary rate of OPPV

In order to determine the evolutionary rate of change for OPPV the TreeRate program with minimum sum of variance optimization was used (Maljkovic Berry., et al 2007) http://www.hiv.lanl.gov/content/sequence/TREERATE/combinedBranchlength.html. Briefly, to determine intra-animal rates of evolution, a tree is rooted in all possible ways and the difference in the average branch lengths between the root and the two time points is calculated. To determine inter-animal rates of evolution, a tree is rooted in all possible ways and the difference in the average branch lengths between the root and the dam and daughter sequences is calculated. The daughter's age was used as the time difference per the conservative assumption that maternal transmission occurred at parturition. The root that gives the minimum sum of variance is the best estimate for the evolutionary rate, and results are given in substitutions site^{-1} year^{-1}. A Student's t-test was used to test for a significant difference in the intra-animal and inter-animal rates of evolution (SAS v. 9.1, SAS Institute, Cary NC).

Other statistical tests

To test for a predominant type of transmission, maternal transmission proportions were analyzed using a binomial test for equal proportions.

CHAPTER THREE

RESULTS

Evaluation of env gene from a previous natural transmission study

Previous work described proviral clearance following postpartum maternal transmission of OPPV (Herrmann-Hoesing et al., 2007a). Briefly, detectable peripheral proviral levels were measured in 22 lambs at 8 weeks of age, but by week 22 of age, peripheral proviral levels were observed only in two lambs, and by 30 weeks were undetectable in all 22 lambs. The lambs remained both serologically and peripheral provirus negative for the next four years. However, an unrelated ewe (112-45) who had previous contact with the OPPV positive dams and was placed with the lambs after the dams had been euthanized became both proviral and serologically positive for OPPV. After the completion of this study we continued to monitor the 22 lambs by serological and molecular means until they were 6 years of age. Subsequently, one of the lambs (LMH35) became OPPV positive by both serological and peripheral proviral assays. In order to determine the source of these two infections we employed a phylogenetic analysis of the proviral envelope (*env*) gene from peripheral blood mononuclear cells (PBMC) and colostrum cells of the 10 original OPPV positive ewes with detectable provirus levels (LMH11, LMH13, LMH15, LMH16, LMH18, LMH19), PBMC of LMH35, and PBMC of 112-45. Figure 1 shows that there are clear transmission links within the maximum likelihood tree between *env* of LMH11 (including both PBMC and colostrum cells) and LMH35 (PBMC) and between *env* of LMH19 (PBMC) and 112-45 (PBMC) with 1000 bootstrap replicate support. The Bayesian tree is concordant to the maximum likelihood tree with 0.99 or greater posterior probability support (data not shown). LMH11 is the dam of LMH35, and LMH19 is unrelated to 112-45. Thus we observed one maternal transmission event and one non-maternal transmission event.

Rates of intra-animal and inter-animal evolution of OPPV env and ltr

Using at least two different time points of OPPV proviral *env* and *ltr* sequences amplified from PBMCs of three unrelated ewes, intra-animal evolutionary rates of 3.9×10^{-3} (95% CI: 2.3×10^{-3} to 5.6×10^{-3}) and 1.6×10^{-3} (95% CI: 0.55×10^{-3} to 2.6×10^{-3}) substitutions site^{-1} year^{-1} were determined for OPPV *env* and *ltr*, respectively (Table 3). These values are significantly different from each other ($P < 0.01$). In addition, inter-animal evolutionary rates of 3.9×10^{-3} (95% CI: 2.7×10^{-3} to 4.8×10^{-3}) and 0.78×10^{-3} (95% CI: 0.36×10^{-3} to 1.2×10^{-3}) substitutions site^{-1} year^{-1} were determined for OPPV *env* and *ltr,* respectively, using the single maternal transmission event from the natural transmission study (Table 3). These values are significantly different from each other ($P < 0.01$).

Rationale for assessing maternal transmission amongst dam-daughter pairs in an OPPV endemic Idaho ewe flock

The results of the natural transmission study of 10 ewes and 22 lambs indicated that there was one maternal transmission event and one non-maternal event. These numbers were insufficient to resolve maternal and non-maternal transmission proportions in this small naturally OPPV infected flock. Therefore, a molecular epidemiological study of a large naturally OPPV infected Idaho ewe flock was utilized to determine the amount of maternal transmission that occurs between dam-daughter pairs. *Env* and *ltr* proviral genes from peripheral blood cells of seropositive OPPV dam-daughter pairs were examined as these genes had previously been described as the two most divergent in the OPPV genome (Zanoni et al., 1998). Sequence analyses methods including two phylogenetic analyses (maximum likelihood and Bayesian) and

pairwise divergence calculations were utilized to determine maternal transmission events from unrelated transmission events in dam-daughter pairs. Forty serologically and peripheral provirus positive dam-daughter pairs were found in a sampling of 1098 ewes; thirty-five were analyzed based on *env*, and forty were analyzed based on *ltr*.

Pairwise nucleotide divergence calculations between dam-daughter pairs and dam-unrelated ewe pairs

To determine if dam and daughter had proviral sequences that were more related to each other than the dam to other ewes, pairwise nucleotide divergence calculations were performed using *ltr* and *env*. Nine out of 35 (25.7%) *env* sequences in the dam-daughter pairs had significantly ($P < 0.0015$) related proviral *env* sequences with a range of divergence spanning 0.5-28%; whereas 26 out of 35 (74.3%) dam-daughter pairs did not have significantly related *env* sequences (Table 4). In comparison, pairwise nucleotide divergence calculations revealed that 7 out of 40 (17.5%) *ltr* sequences in the dam-daughter progeny sets had significantly ($P<0.001$) related *ltr* sequences with a range of divergence spanning 0-13%; whereas 33 out of 40 (82.5%) dam-daughter pairs did not have significantly related *ltr* sequences (Table 4). Maternal transmission proportions observed based on pairwise nucleotide divergence calculations for *env* and *ltr* resulted in significant deviations from equality ($P<0.005$ and <0.0001 respectively).

Phylogenetic analysis of OPP env and ltr proviral sequences obtained from dam-daughter pairs

Bayesian and maximum likelihood phylogenetic analyses using *env* and *ltr*, both individually or concatenated were conducted on 35 to 40 OPPV positive dam-daughter pairs. Maternal transmission was defined as dam-daughter *env* or *ltr* proviral sequences which formed

a monophyletic group with ≥ 0.95 posterior probability in Bayesian analysis or ≥ 70% bootstrap support in maximum likelihood analysis. Using this definition, the maximum likelihood phylogenetic analysis determined that 5 out of 35 (14.3%) dam-daughter pairs had related proviral *env* sequences with 100% bootstrap replicate support (Figure 2). Conversely, 30 out of 35 (85.7%) dam-daughter pairs had proviral *env* sequences with more than one common ancestor separating them in the tree. In addition, maximum likelihood phylogenetic analysis showed that 5 out of 40 (12.5%) dam-daughter pairs had related proviral *ltr* sequences with >80% bootstrap replicate support (Figure 3). Four of these dam-daughter pairs identified as maternal transmission events using *ltr* were concordant to 4 of 5 of the dam-daughter pairs identified as maternal transmission events using *env* (Table 4). In addition, further maximum likelihood phylogenetic analysis was performed using a concatenated data set combining both the *env* and *ltr* proviral sequences for 40 dam-daughter pairs (Figure 3). This combined analysis showed 5 out of 40 (12.5%) dam-daughter pairs had related proviral *env* and *ltr* sequences with >78% bootstrap replicate support. These 5 dam-daughter pairs identified as maternal transmission events using both *env* and *ltr* were identical to the 5 dam-daughter pairs identified as maternal transmission events using *env* individually and concordant with 4 of the 5 dam-daughter sets identified in the *ltr* analysis.

Branch lengths between dam-daughter sets from the maximum likelihood *env* tree were used to calculate the amount of nucleotide substitutions site $^{-1}$ or rate of evolution between the dam-daughter sets. The calculated rates for dam-daughter sets were compared against the previously calculated intra-animal and inter-animal rates of evolution (Table 3). The five dam-daughter sets identified as maternal transmission events in the *env* and concatenated phylogenetic analysis have rates of evolution that fall within the 95% confidence interval for the previously

calculated intra-animal and inter-animal nucleotide substitution rates. None of the dam-progeny sets identified as non-maternal transmission events in the *env* and concatenated phylogenetic tree fall within the intra-animal or inter-animal nucleotide substation rate 95% confidence interval.

Bayesian trees were also generated and results were concordant with the observed maximum likelihood trees for *env*, *ltr,* and the combined *env* and *ltr* data set (data not shown). The same criteria of forming a monophyletic group and ≥ 0.95 posterior probability were used to characterize maternal transmitting events in the Bayesian trees. The maternal transmission proportions derived from the *env*, *ltr* and combined *env* and *ltr* phylogenetic analyses resulted in significant deviations from equality ($P<0.0001$).

Defining "true" maternal transmission events based on sequence analyses

To consolidate the different findings between the sequence analyses, a definition was set where two sequence analyses methods were required to agree using *env* or *ltr* in order to be considered as a true maternal transmission event. In this respect, maternal transmission occurred in 5 out of 35 (14.3%) dam-daughter pairs using *env* and 4 out of 40 (10.0%) dam-daughter pairs using *ltr*. Therefore, if this range is chosen for "true" maternal transmission events, then non-maternal transmission accounts for 85.7-90% between dam-daughter pairs in this Idaho ewe flock.

CHAPTER FOUR

DISCUSSION

Using 2 out of 2 sequence analyses methods on a large Idaho ewe flock, "true" maternal transmission accounts for 10-14.3% of OPPV transmission. Conversely horizontal transmission is the predominant type of transmission observed ($P<0.0001$) and accounts for 85.7-90% between dam-daughter pairs in this Idaho ewe flock. This "true" maternal transmission value is similar to the 18-19% of lambs maintaining serological positive status after 10 months post-parturition following natural suckling of and exposure to their dams for 5-6 weeks post-parturition (Alvarez et al., 2005). However, in our first study, only 1 out of 22 dam-progeny pairs (LMH11 and LMH35) or 4.5% showed maternal transmission using molecular epidemiological based on *env* after 6 years post-parturition. Since the original study utilized ewes from the same Idaho flock, the reason for this extremely delayed seroconversion and provirus detection is unknown. However it suggests that maternal transmission is not very efficient under certain management and environmental conditions.

This present study was unable to separate maternal transmission into its component parts of *in utero*, intrapartum, and postpartum (lactogenic and contact) transmission. However, earlier reports suggest that *in utero* maternal transmission occurs 5% of the time, and intrapartum maternal transmission does not appear to contribute significantly to overall maternal transmission (Cutlip et al., 1981). Therefore, if "true" maternal transmission occurs in 10-14.3% of cases and *in utero* maternal transmission accounts for 5%, then the remaining 5-9.3% of maternal transmission may be due to maternal contact and lactogenic transmission. The low amount of maternal transmission in this flock could be due to high amounts of anti-OPPV antibody in the colostrum/milk of ewes, which may control the amount of cell-associated and

cell-free virus transmitted via the colostrum/milk (lactogenic) and respiratory secretions (contact) to a level that cannot cause persistent infection (Herrmann-Hoesing et al., 2007).

Non-maternal transmission accounts for 85.7-90% OPPV transmission within this ewe flock which has no control programs in place. There could be various non-maternal transmission routes occurring in this ewe flock. Prior research has shown that if colostrum/milk from a MVV infected dam is fed to an unrelated lamb or if colostrum/milk is pooled from several CAEV infected dams and fed to several kids, transmission events increase as compared to lambs or kids with contact and colostrum/milk feeding from their individual dams (Adams et al., 1983; Alvarez et al., 2005). These findings are reinforced by the fact that non-maternal transmission through milk aerosolization may also contribute significantly to overall transmission in CAEV infected goat dairies (Rowe and East, 1997). In addition, intensive operations with high milk producing sheep breeds have increased seroprevalence as compared to extensive operations with lower milk producing sheep breeds in Spain (Leginagoikoa et al., 2006). These combined results suggest that the type of management situation (i.e. dairy/intensive and meat-wool/extensive) or the breed of animal may dictate the amount of lactogenic maternal and non-maternal transmission in a flock.

It is unknown how much respiratory secretions contribute to overall transmission of OPP. However, since cell-associated and cell-free virus is found in bronchial alveolar lavage fluid, it is believed that respiratory secretions contribute to OPP transmission (Lujan et al., 1994; Brodie et al., 1995; McNeilly et al., 2008). Feces may also play a role in transmission since naïve sheep developed MVV following oral inoculation of feces from MVV infected animals (Sigurdsson et al., 1953). Additionally, iatrogenic routes such as needle re-use during vaccinations and man-made routes such as shearing, tail-docking and castration cannot be excluded as possible vehicles

of transmission. Sexual and paternal transmission of OPPV have not been demonstrated, but the presence of cell associated and cell free virions in the semen of infected rams during concurrent *B. ovis* infections suggests that these types of transmission may occur (de la Concha-Bermejillo et al., 1996; Peterson et al., 2008).

Using serial proviral sequence time points, this paper is the first to report the evolutionary rate of change of OPPV within an animal and between animals. The observed rate of change for both *env* and *ltr* is within range of the substitution rate calculated for other RNA viruses (Jenkins et al., 2002, Hanada et al., 2004). The intra-animal evolutionary rate of change calculated for OPPV proviral *env* of 3.9×10^{-3} substitutions site^{-1} year^{-1} is comparable to what has been described for HIV proviral *env* (Li et al., 1988; Leitner et al., 1999; Korber et al., 2002; Salazar-Gonzalez et al., 2008). Interestingly, the observed *ltr* inter- and intra-animal rate of change is slower than in *env* for OPP. In HIV-1 it has been proposed that the increased substitution rate of the *env* V3 region indicates diversifying selection on the region while the lower substitution rates of *gag* and *pol* are probably due to purifying selection (Leitner et al., 1999; Korber et al., 2000).

Inter-animal substitutions/site or rates of evolution based on branch lengths in the envelope and concatenated maximum likelihood phylogenetic trees for the 5 maternal transmission events were within the 95% confidence interval for intra-animal and inter-animal rates of evolution. However, rates based upon the branch lengths for the 30 non-maternal transmission events were higher than the maximum values for the inter-animal or intra-animal substitution rate 95% confidence interval. Along with this, inter-animal evolution rates (substitutions/site/year) were calculated for the "true" maternal transmission events (data not shown), and these rates were also within the 95% confidence interval for the intra-animal and inter-animal substitution rates of evolution. These data suggest the number of maternal

transmission events has not been underestimated due to the accumulation of a large number of nucleotide substitutions within an animal's lifetime or between dam and daughter viral strains since the initial transmission event. In addition, these data suggest that there is not a large accumulation of nucleotide substitutions after maternal transmission, and this could be due to the co-adaptation of the virus strain with maternal immunogenetics.

In contrast to previously published results (Zanoni 1998) we found that the *ltr* was more highly conserved than the *env* gene with maximum divergence values being 13% and 28% for *ltr* and *env*, respectively, within this dam/daughter flock. The *ltr* contains the promoter and enhancer regions, and these regulatory regions may account for *ltr* being more highly conserved than *env*. In addition, "true" maternal transmission in the Idaho ewe flock was lower using *ltr* than *env* (10.0% versus 14.3%), and this lower value for *ltr* may reflect its increased conservation over *env*. As a side note, the neurological form of MVV seems to be controlled by a 54-bp repeat found in the *ltr* (Oskarsson et al., 2007); however, this repeat was not observed within the *ltr* of any of these Idaho ewes.

In HIV-1 it has been shown that increased levels of plasma viremia and mother-child MHC homozygosity both associate with increased maternal transmission rates (Borkowsky et al., 1994; Garcia et al., 1999; Ioannidis et al., 2001; Polycarpou et al., 2002; Mackelprang et al., 2008). Both *env* proviral load and MHC class II DRB1 genetics between transmitting and non-transmitting dam-daughter sets were evaluated, however, we could find no association between either proviral levels or MHC class II DRB1 genetics with the small numbers of maternally transmitting pairs (4 pairs for *ltr* or 5 pairs for *env*) identified in this study (data not shown). Larger numbers of true maternal transmission events need to be evaluated in order to fully assess whether proviral load and MHC class II DRB1 genetics play a role in maternal transmission.

In conclusion, we have presented strong sequence and phylogenetic evidence that maternal transmission, while still important, plays only a minor role in the spread of OPPV within a naturally infected ewe flock. Future molecular epidemiological studies on dam-daughter pairs need to be conducted on other flocks of different breeds and production systems to assess whether this flock is unique to low maternal transmission events. Molecular epidemiology studies on paternal lines and the ewe flock may elucidate the contribution of paternal transmission and sexual transmission routes to overall OPPV transmission.

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Table 1. List of oligonucleotide primers

Designation	Sequence (5' - 3')	Polarity[a]	Location[b]	PCR[c]
Envelope PCR				
ENV1	CCAGGAGGATTTCAGARRGT	S	363 - 382	1
TMcon	CCGTCCTTGTGTAGGATTGCT	A	1998-2002	1&2
ENV87	AAACTTTACTCAATGGGGGTGTCA	S	421-445	2
LTR PCR				
LTR1	CAGGACAGAGAGCAAATGCCT	S	1-22	1
GAGLTR1	TTCCTTAAGCTCGGGGTATCC	A	763-784	1&2
LTR2	RTGTCATTGTTACCAGAAAG	S	71-91	2
Envelope Sequencing				
ENV683	ATGGGGGAATAAAAGATAGAAAT	S	1017-1040	
ENV1048	TATGTTGTGTCTTTCTGGCCTCTG	A	1382-1406	

[a]Signifies sense (S) or antisense (A) directions.
[b]Nucleotide position for reference strain 85/34 U64439 for *env* and AY101611 for *ltr*.
[c]Refers to use in first (1) or second (2) round of PCR.

Table 2. Average base composition for analyzed genes.

Gene	A	C	G	T	α^b	Invariants
env	38.2 (0.4)[a]	15.2 (0.2)	25.7 (0.3)	20.7 (0.2)	0.446	0.302
ltr	31.8 (0.5)	20.3 (0.3)	29.1 (0.4)	18.8 (0.3)	0.548	0.429

[a]Values in brackets indicate standard deviation.

[b]Alpha estimates <0.7 indicate strong among site variation.

Table 3.Average evolutionary rates for *env* and *ltr* gene fragments

Type	Gene	No. of sequences	No. of trees[a]	Spanning years	Rate[b]	95% C.I.
Intra	*ltr*	50	8	2002-2008	1.6	0.55 - 2.6
Intra	*env*	56	5	2002-2007	3.9	2.3 - 5.6
Inter	*ltr*	57	10	2002-2008	0.78	0.36 - 1.2
Inter	*env*	85	7	2002-2007	3.9	2.7 - 4.8

[a]Number of phylogenetic trees used to calculate substitution rate
[b]Rate given in 10^{-3} substitutions $site^{-1}$ $year^{-1}$

Table 4. Dam-daughter (maternal) OPPV transmission events (and percentage) in an ewe flock using two different sequence analyses methods for provirus *env* and *ltr*

	env (n=35)		*ltr* (n=40)	
	Phylogenetic analyses[a]	Pair wise divergence	Phylogenetic analyses	Pair wise divergence
Daughter ID				
H1549	N[b]	P	N	N
H2194	N	P	N	P
H2695	**P**	**P**	**P**	**P**
H2823	**P**	**P**	**P**	**P**
H3396	**P**	**P**	**P**	**P**
K3749	N	P	N	P
K3994	N	N	P	N
R6139	N	N	N	P
R6326	N	P	N	N
R6333	**P**	**P**	**P**	**P**
R6800	**P**	**P**	N	N
Total	5 (14.3%)	9 (25.7%)	5 (12.5%)	7(17.5%)

[a]Based on maximum likelihood and Bayesian methods. Concatenated *env* and *ltr* phylogenetic analyses had identical results as *env* phylogenetic analyses results.
[b]P indicates a positive result and N indicates a negative results for a dam-daughter transmission event using the given sequence analysis method.
Bold P's indicate a positive result for the dam-daughter pair using all methods for provirus *env* and *ltr*.

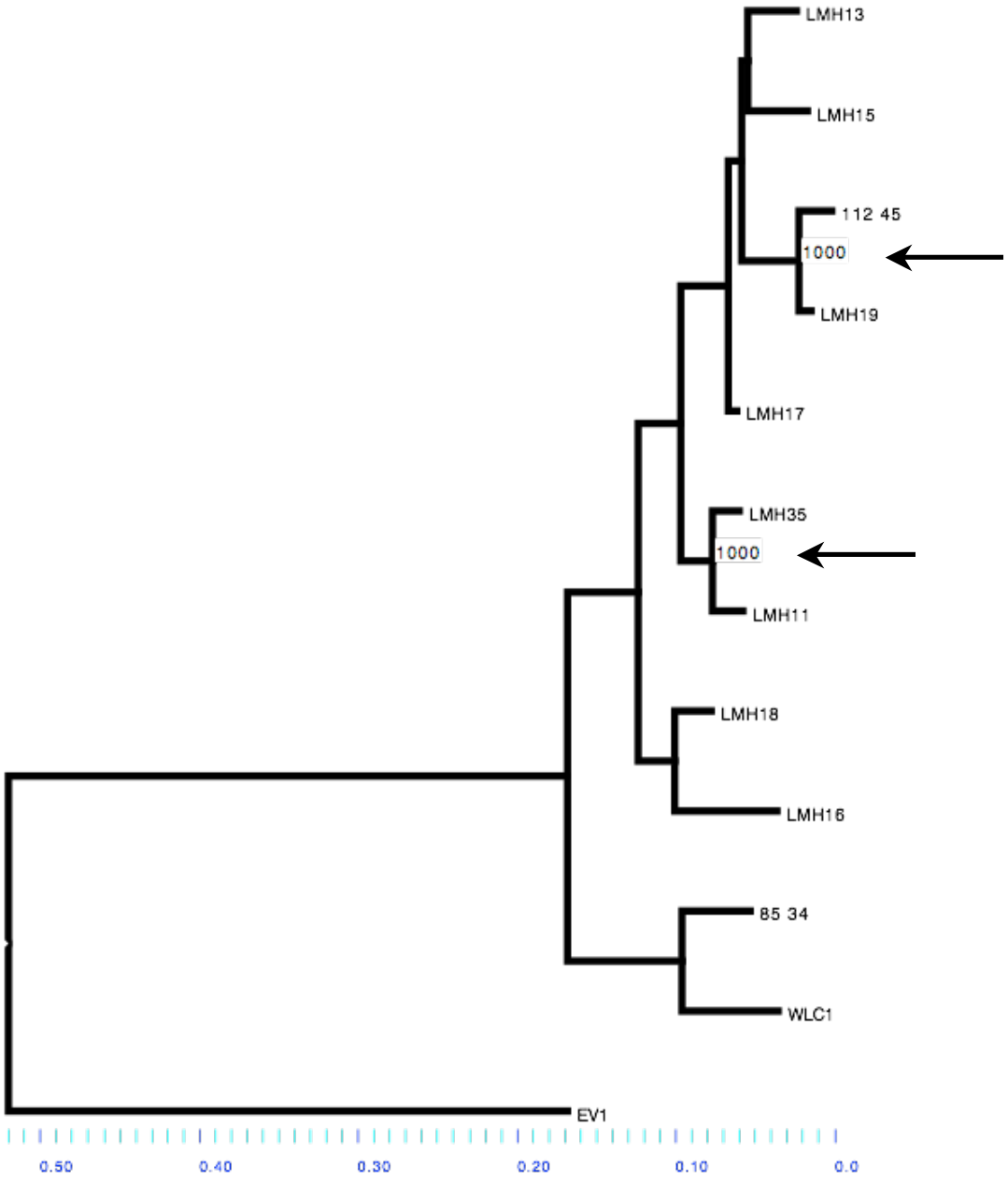

Figure 1. Maximum likelihood phylogenetic analysis of *env* in small OPPV infected flock. Branch support is measured out of 1000 bootstraps. The tree was outgroup rooted with the MVV strain EV1. Arrows indicate transmission events with corresponding bootstrap values.

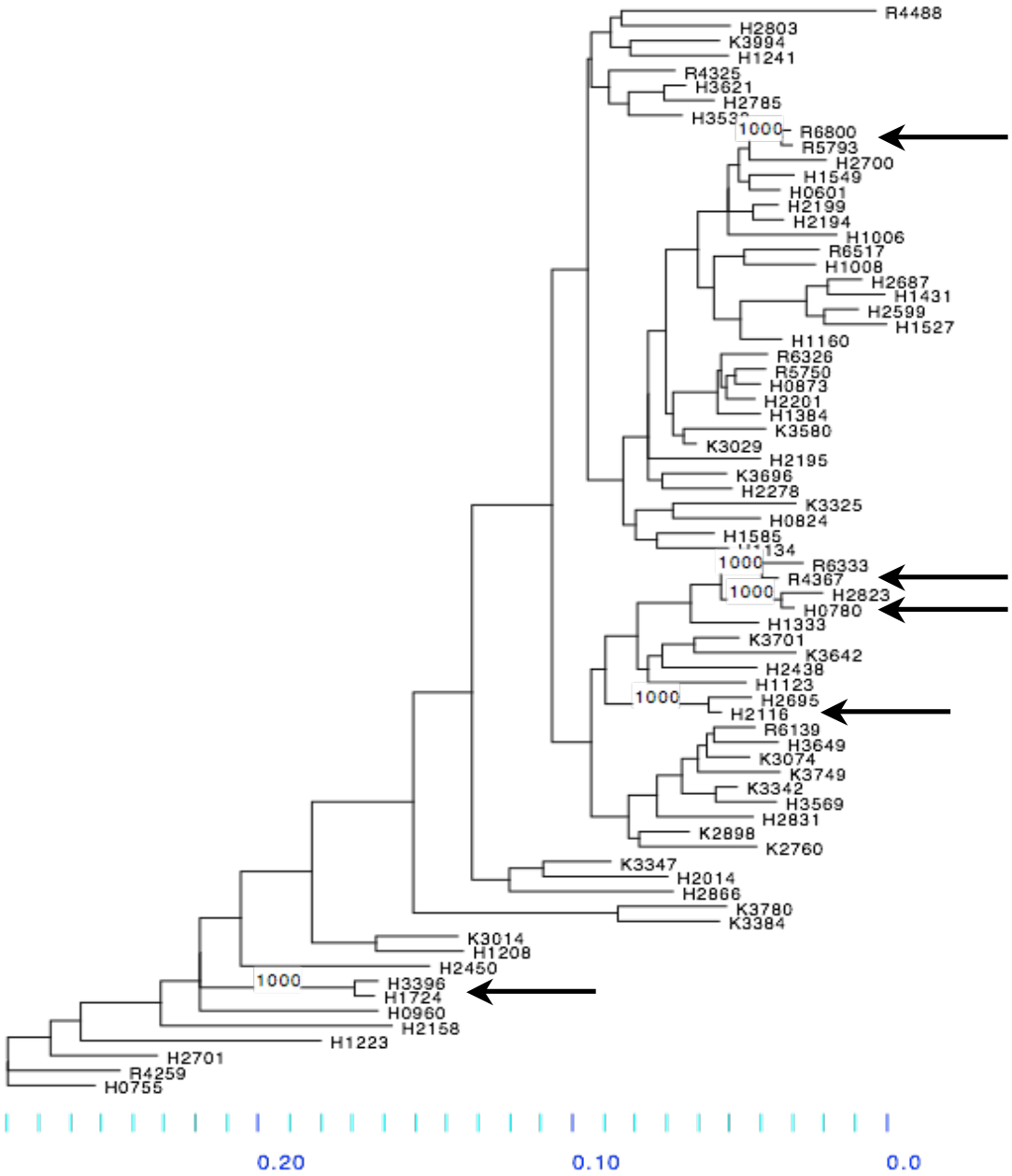

Figure 2. Maximum likelihood phylogenetic analysis of *env* in 35 OPPV positive dam-daughter pairs from a large OPPV infected flock. Branch support is measure out of 1000 bootstraps. Arrows indicate maternal transmission events with corresponding bootstrap values.

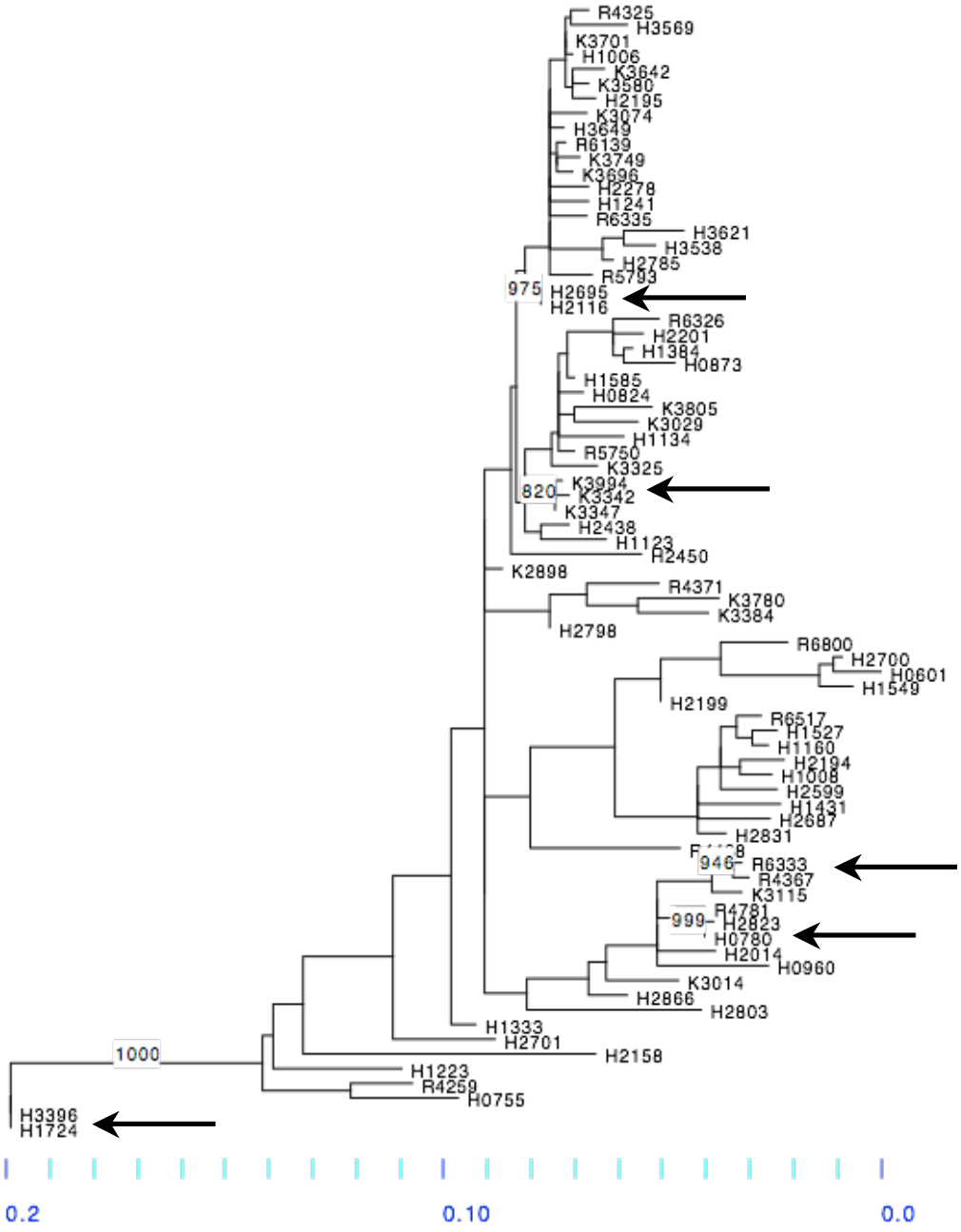

Figure 3. Maximum likelihood phylogenetic analysis of *ltr* in 40 OPPV positive dam-daughter pairs from a large OPPV infected flock. Branch support is measure out of 1000 bootstraps. Arrows indicate maternal transmission events with corresponding bootstrap values.

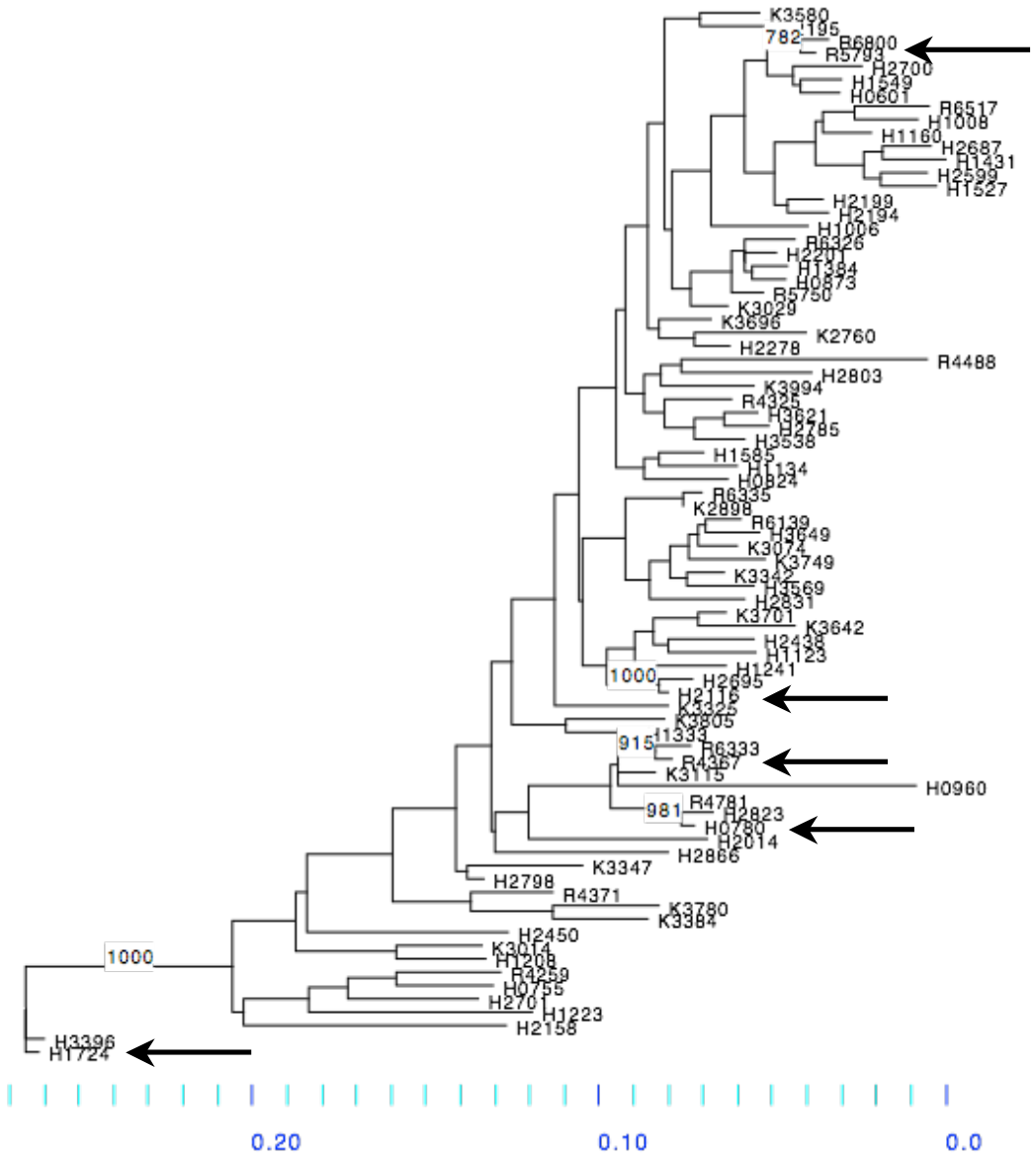

Figure 4. Maximum likelihood phylogenetic analysis of combined *env* and *ltr* in 40 OPPV positive dam-daughter pairs from a large OPPV infected flock. Branch support is measure out of 1000 bootstraps. Arrows indicate maternal transmission events with corresponding bootstrap values.