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# Genomic analysis of bovine herpesvirus type 4 (BoHV-4) from Argentina: high genetic variability and novel phylogenetic groups

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## Summary

Bovine herpesvirus 4 (BoHV-4) is a γ-herpesvirus that has been isolated both from apparently healthy animals and from cattle with a variety of clinical signs, including post-partum endometritis and abortion. BoHV-4 causes either a persistent or a latent infection in cells of the monocyte/macrophage lineage. Two groups of BoVH-4 strains have been

defined based on their restriction patterns: the Movar-like strains (European prototype) and the DN 599-like strains (American prototype). The purpose of the present study was to genetically characterize wild type BoHV-4 strains isolated from vaginal discharges of aborted cows in Argentina. The virus was identified by isolation and nested PCR in all vaginal discharge samples from aborted cows, either as a sole agent or in association with other pathogens. Restriction enzyme profiling and phylogenetic analysis demonstrated that there is a high genetic variability among the studied field isolates. The existence of three groups of strains, which were designated as genotypes 1, 2 and 3, is described. Genotypes 1 and 2 possibly correspond to the Movar-like and DN 599-like groups, respectively, whereas Genotype 3 corresponds to a novel group. Two viral strains did not cluster into any of these three groups, indicating that other genotypes could be circulating in Argentina. These results suggest a complex epidemiological background for the Argentinean BoHV-4 strains, probably influenced by independent events of genetic drift. This hypothesis cannot be rejected based on the available data. However, there is no direct evidence supporting this possibility. Thus, it seems speculative to suggest that interspecific jumps are responsible for the observed phylogenetic grouping. On the other hand, our analyses suggest a geographical structure for the observed viral genotypes, since genotypes 1 and 2 included the European (Movar-like) and American (DN599-like) reference strains, respectively. Geographic dispersion is known to be a driver of herpes viruses diversification, and independent evolution in geographical isolated places ensures the emergence of particular mutations in each location due to genetic drift (Current topics in microbiology and immunology 312:1-42; Journal of virology 73:4156-4170). Therefore, at this point, the genetic drift hypothesis is the one that requires less ad-hoc considerations and thus, to our

understanding, is the one that fits to the findings from this study. The involvement of this genetic variability in the detection and pathogenesis of BoHV-4 remains to be investigated.

**Keywords**: Bovine herpesvirus type 4; Aborted cows; Phylogenetic analysis; Restriction endonuclease

## Introduction

Bovine herpesvirus 4 (BoHV-4) belongs to the family *Herpesviridae*, subfamily *Gammaherpesvirinae* and genus *Rhadinovirus*. BoHV-4 has no close biological or virological relationship to other known herpesviruses of the family *Bovidae* (Bartha *et al.*, 1966; Fabian *et al*., 2008). Cattle are the natural hosts of the virus. However, several ruminant (Dewals *et al*., 2006) and non-ruminant species are also susceptible to BoHV-4 (Egyed *et al.*, 1997). Although replication of most γ-herpesviruses is restricted to their natural host species, BoHV-4 is highly promiscuous. Sporadic isolates from species as diverse as lions, cats and owl monkeys have also been described (Barahona *et al*., 1973; Bublot *et al*., 1991). BoHV-4 has also been reported to infect goats (Moreno-Lopez *et al.*, 1989), guinea pigs, and rabbits (Egyed *et al*., 1997). Furthermore, BoHV-4 replicates in animal as well as in human cell lines, for example, human embryonic lung cells and HeLa cells (Truman *et al*., 1986; Egyed *et al*., 1998).

The virus has been isolated from apparently healthy animals (Storz *et al*., 1984) as well as from cattle with a variety of clinical signs, including diarrhea, metritis (Frazier *et al*., 2002; Izumi *et al*., 2006; Nikolin *et al*., 2006), abortion, vaginitis, mastitis (Miyano *et al*., 2004), ulcerative mammillitis and skin lesions (Goyal and Naeem, 1992). However, relevant information concerning the involvement of BoHV-4 has been obtained only from

relatively few cases (Evermann *et al*., 1985). This lack of information is due to the inherent difficulties in the diagnosis of BoHV-4 infections, since rapid diagnostic methods other than direct examination of the infected animal are not available (Ludwig, 1982; Osorio *et al*., 1985).

Like other herpesviruses, BoHV-4 has been isolated from persistently infected cattle (Dubuisson *et al*., 1989). Persistent infection is characterized by the constant presence of circulating antibodies. However, these antibodies are not fully protective against re-infections. Latency in lymphoid tissues and prolonged viremia associated with mononuclear cells has been demonstrated (Osorio *et al*., 1983). The latent virus can be reactivated by stress factors or glucocorticoid treatment (Dubuisson *et al*., 1989).

The role of BoHV-4 in the infection of the genital tract is being studied by several research groups. The virus has been reported to be responsible for post-partum and chronic metritis, alone or in combination with other pathogens (Wellemans *et al*., 1986; Monge *et al*., 2006). Recently, BoHV-4 has been isolated from three vaginal discharge samples from cows with post-partum metritis, some of which had BoHV-4-specific neutralizing antibodies (Nikolin *et al*., 2007).

In 2007, BoHV-4 was isolated in Argentina from samples of bovine abortions (Verna *et al*. 2008a) and later, from nasal swabs, brain tissue, oocytes, and granulosa cells and from semen from an artificial insemination center. We also isolated the virus from buffy coat fractions in association with bovine viral diarrhea virus (BVDV) (Unpublished).

All BoHV-4 strains analyzed so far exhibit close, similar restriction profiles and a well conserved central part of the genome. However, the prDNA (called polyrepetitive DNA) region varies in size depending on the number of repetitions of a 200 bp fragment

(Ehlers *et al*., 1985). Two groups of BoVH-4 strains have been defined based on their restriction patterns: the Movar 33/63- like (Movar-like) strains and the DN 599-like (DN-like) strains. The DN-like group encloses all the American strains, the European strains isolated in the USA from bison and cats, and the African strain isolated from buffalo. All the other known European strains belong to the Movar-like group (Thiry *et al*., 1990). Using cross-hybridization with the DN599 reference strain, Henry *et al*. (1986) analyzed eight American isolates from cattle with various diseases and found that only one of the isolates had a restriction pattern clearly different from the reference strain. Thus, they could not establish a correlation between the origin and the restriction pattern of the isolates. Frazier *et al*. (2002) stated that BoHV-4 is capable of significant antigenic drift and suggested that endometriotropic strains might arise from less pathogenic or non-pathogenic BoHV-4 strains. Nevertheless, variations occurring in the genome of BoHV-4 field isolates have not been documented.

In a previous work, we isolated BoHV-4 from one out of eleven peripheral blood leukocyte (PBL) samples from one herd (Peréz *et al*., 2011). By restriction endonuclease analysis (REA), we demonstrated the existence of genomic variation among the strains circulating in this herd. We also found that the restriction patterns of the BHV-4 genomes present in all the PBL samples evaluated differed from those of the American prototype strain DN 599. Overall, this work demonstrated that BoHV-4 is present in the leukocyte fraction of dairy cattle and that viral strains present in this herd are genetically divergent.

Although BoHV-4 has been isolated from animals with different clinical manifestations as well as from healthy animals, the relationship between biotypes of BoHV-4 and uterine disease has not been explored. The purpose of the present study was to genetically

112 characterize wild type BoHV-4 strains isolated from vaginal discharges of aborted cows in
113 Argentina

114 ***Methods***

115 ***Cell culture and viral isolates***

116 Madin Darby bovine kidney (MDBK) cells cultured in minimum essential medium
117 (MEM) supplemented with 10% fetal bovine serum were used for BoHV-4 propagation
118 Cells were provide by the ABAC (Argentinean Cell Bank) and they are certified free of
119 contaminating bacteria, mycoplasma and adventitious viruses. Seventeen BoHV-4 strains
120 were obtained from vaginal discharge samples of aborted cows. Samples were inoculated in
121 triplicate in 96 well-plates and observed daily for the presence of cytopathic effect (CPE).
122 Blind passages were performed every 48 hours, and at the third blind passage, samples
123 were routinely tested for the presence of bovine herpesvirus 1 (BoHV-1) and BVDV.
124 Because BoHV-4 does not replicate easily in cell cultures, samples were maintained in
125 MDBK cells for up to ten blind passages. When CPE was observed and the presence of
126 BoHV-4 was confirmed by nested PCR, the viral stock was amplified and stored at -80°C.
127 The origin, year, and location of BoHV-4 isolates, as well as the clinical condition and age
128 of the animal from which the virus was identified are detailed in Table 1. Strains belonging
129 to the DN 599-like group (North American prototype) were used as reference viruses for
130 REA, nested PCR and phylogenetic analysis.

131 ***Extraction of viral DNA and PCR***

DNA was extracted from infected cells using a commercially available kit (DNeasy blood & tissue Kit, Cat. 69504, Qiagen), according to the manufacturer's instructions. DNA concentration was determined by spectrophotometry at an absorbance of 260 nm. Nested PCR was performed as previously described (Verna *et al*., 2008), using primers that amplify the thymidine kinase (TK) gene of BoHV-4. For the first amplification round, 3 µl of DNA was added to the PCR reaction mix (25 µl final volume), containing 0.2 µM of each primer, 200 µM dNTPs, 25 mM $MgCl_2$ and 1 U DNA polymerase. Primer sequences for the first PCR round are: 5´-GTTGGGCGTCCTGTATGGTAGC-3´; 5´-ATGTATGCCCAAAACTTATAATATGACCAG-3, and the amplification product is 567 bp. Primer sequences for the second PCR round are: 5´-TTGATAGTGCGTTGTTGGGATGTGGT-3´ and 5´-CACTGCCCGGTGGGAAATAGCA-3´ and the amplification product is 260 bp. Amplification was carried out as follows: 95°C 9 min 94° C 45s; 58° C 60s; 72° C 90s for 20 cycles and one extension cycle at 72° C for 7 min. For the second amplification round, 2.5 µl of the first round PCR product was used and the annealing temperature was decreased to 55° C. Mock-infected MDBK cells were used as negative control. Amplification products were not obtained when DNA from the BoHV-1 and BoHV-5 reference strains were included in the PCR reactions. PCR products were visualized on 1.5% agarose gels stained with ethidium bromide. The obtained amplicons were sequenced and the corresponding sequences were deposited in GenBank under accession numbers JQ838046 to JQ838062.

***Restriction endonuclease analysis***

Viral DNA was digested by restriction endonucleases. An aliquot of 5 µg of extracted viral DNA was digested, at 37 °C with 20 U of *Eco*R1, *Bam*H1 and *Hin*dIII (Promega). The digested product was subjected to electrophoresis at 15 V on a 0.8% agarose gel, using TAE (Tris-acetate-EDTA) electrophoresis buffer and visualized with ethidium bromide. Lambda DNA *Hin*dIII fragments were used as molecular weight markers.

***Phylogenetic analysis***

The sequences were aligned using the *Mafft* program (Katoh *et al.,* 2002). Phylogenetic trees were inferred with the MrBayes program (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The nucleotide evolutionary model (GTR) was inferred by the MrAIC.pl script (Nylander, 200). Eight Markov chain-Monte Carlo (MCMC) chains were run for 10E7 generations, sampling every 1000 generations. Adequate mixing and convergence were assessed by the Tracer program (Rambaut and Drummond, 2007).

***Results***

**Identification of pathogens in aborted cows**

As described in Table 1, the BoHV-4 isolates analyzed in this study were obtained from vaginal discharge samples of aborted, adult cows. Fetuses that were recovered after abortion ranged between 4 and 8 months of gestations. In most cases, the etiological agent responsible for the abortion could not be identified. BVDV, *Histophilus somni* and *Arcanobacterium pyogenes* were detected in the vaginal discharges of aborted cows, in

combination with BoHV-4. The presence of BoHV-1 was investigated in all vaginal discharge samples. However, this virus was not identified by co-cultivation on MDBK cells and direct immunofluorescence (data not shown).

Serum antibody titers to *Neospora caninum* and *Leptospira* spp. were detected in some aborted animals. In agreement with other reports (Deim *et al*., 2006; 2007), even in those cases in which BoHV-4 was the only pathogen identified, it was not possible to demonstrate that the virus was the only agent responsible for the abortion.

A BoHV-4-specific 260-bp fragment was amplified from all the vaginal discharge samples analyzed.

**Enzyme restriction patterns of BoHV-4 isolates**

The *Eco*R1, *Bam*H1 and *Hind*III genomic restriction patterns of eighteen BoHV-4 field isolates digested with are shown in Figs 1, 2, and 3, respectively. A high variability was evident among the REA patterns of the different isolates. In general, variations in the number of restriction sites after digestion with any of the three enzymes were remarkable; not only among isolates but also with respect to the reference strain DN 599.

The fragment patterns generated after *Eco*R1 digestion were highly heterogeneous, except for isolates 5 and 10 and, 3, 6 and 9 [Fig. 1 (07/435; 09/759 and 08/476; 08/433 and 07/579)]), which showed similar restriction profiles. By sequencing analysis, we were able to determine that isolates 3, 6, 9 and 10 belong to the DN-like group, and that isolates 5 and 09/227 belong to a different genotype, designated as Genotype 3 (or MGA-like group). All

the vaginal discharge samples evaluated with *Eco*R1 differed from the American prototype strain DN 599 (Fig. 1).

A similar degree of variation in the restriction patterns was observed among isolates after *Hin*dIII digestion (Fig. 2). Only two BoHV-4 strains (08/209 and 08/476) had a REA pattern similar to that of the reference strain DN 599. Nevertheless, these isolates had three additional fragments. Isolates 4, 7 and 8 were also similar, although isolate 8 had an extra fragment in comparison to isolates 4 and 7.

The *Bam*H1 restriction endonuclease profiles after digestion are shown in Fig. 3. The number and sizes of the fragments were distinct among the isolates. The profiles of isolates 8 and 10 were similar to that of DN 599. Although the REA patterns for isolates 7 and 9 were similar to those of isolates 8, 10 and strain DN 599, an extra fragment was observed. A similar profile between isolates 2 and 3 was also detected.

The restriction patterns of the DN 599-like, 08/404 and 08/415 strains are presented in Fig.4. In relation to the other BoHV-4 isolates analyzed in this study, these two field strains showed fewer restriction sites, as observed after digestion with the three endonucleases.

**Phylogenetic relationship of BoHV-4 Argentinean field isolates**

Sequences from the BoHV-4 TK gene were used to study the phylogenetic relationships and clustering patterns of the viral strains identified in the vaginal discharges of the seventeen aborted cows that had been previously characterized according to their restriction profiles (Table 1). These analyses revealed the existence of three strongly

supported groups of strains (Fig. 5), which were designated as *Genotype 1*, *2* and *3*. Genotypes 1 and 2 possibly correspond to or are closely related to the previously described European (Movar-like) and American (DN599-like) groups, respectively, as the corresponding reference strains (Movar_33_63 and DN 599) clustered into these clades (Fig. 5). By contrast, Genotype 3 seems to have no counterparts among any previously described group. The inability of strains 08/415 and 08/404 to cluster into any of these three genotypes (Fig. 5) and the fact that they also displayed unique and particular restriction profiles (Fig. 4), support the idea that these strains could belong to two extra genotypes. Isolates 08/415 and 08/404 have different geographic origins. Furthermore, both isolates come from cases in which no other abortigenic pathogens were isolated, which suggest that the viral genetic background could influence the clinical outcome of the infection.

## *Discussion*

Bovine herpesvirus type 4 is one of the five groups of herpesviruses associated with cattle. BoHV-4 has also been referred to as bovine cytomegalovirus because of its slow growth and marked cell association (Ludwig, 1982; Storz *et al*., 1984). The real frequency of BoHV-4-induced disease in cattle is unknown. However, the virus is prevalent in bovine populations, and, under circumstances of host immunosuppression, it replicates actively, as judged by the presence of BoHV-4-specific antibodies (Evermann *et al*., 1985).

BoHV-4 infection has been involved in reproductive tract disorders and viral pathogenicity has been related, in part, to the viral strain involved (Castrucci *et al*., 1987). In this study, the virus was identified by isolation and nested PCR in all vaginal discharge samples from

238 aborted cows, either as a sole agent or in association with other pathogens. The
239 identification of pathogens as a potential cause of abortion was evident in nine of the cases
240 analyzed in this study. Specifically, *Leptospira*, *N. caninum*, *Arcanobacterium pyogenes*,
241 *Histophilus somni* and BVDV can directly or indirectly be implicated as the cause of
242 abortion. However, many of these agents have been described as post-partum invaders of
243 the uterus, and this may be a reason why isolation from vaginal discharges is also frequent.
244 On the other hand, detection of BoHV-4 as a sole agent is an indirect evidence of the
245 involvement of the virus in bovine abortion.

246 The pathogenesis of BoHV-4 infection has been questioned due to the isolation of
247 BoHV-4 from healthy individuals, and from cattle with a wide variety of clinical signs
248 (Wellenberg *et al*., 2000; Monge *et al*., 2006). The virus has been isolated from cattle with
249 genital disorders, such as metritis. Several studies have established a strong relationship
250 between the antibody response to BoHV-4 and the presence of fertility problems
251 (Wellemans *et al*., 1986; Czaplicki *et al*., 1998; Monge *et al*., 2006). In contrast to the
252 findings by Fabian *et al*. (2008), Frazier *et al*. (2002) and Monge *et al*. (2006) reported that
253 cases of post-partum metritis associated with BoHV-4 are an important problem in the USA
254 and Spain, respectively.

255 In the present study, the virological examination of BoHV-4 infection was
256 performed using cell culture and TK nested PCR. A nested PCR for the detection of TK
257 genomic sequences has been previously described by Egyed *et al*. (1996). This assay has
258 been shown to be suitable to study the *in vivo* distribution of BoHV-4 in its host. Here, we
259 confirmed the findings that the selected primers for the amplification of the BoHV-4 TK
260 gene are specific (Egyed *et al*., 1996). A potential advantage of the PCR assay over virus

isolation is that BoHV-4 DNA can be detected in different samples despite the absence of BoHV-4 cytopathic effect.

The nucleotide sequence of the PCR-amplified TK gene fragment of 17 BoHV-4 strains was compared with the DN 599 sequence of BoHV-4, which is published and stored in GenBank (S49773.1). Restriction profiles of BoHV-4 DNA completely differ from those of other types of BoHV (Osorio *et al*., 1985). Although the profiles among BoHV-4 isolates are closely related, BoHV-4 strains were divided into two groups according to the patterns of the digested DNA (Thiry *et al*., 1989). This classification is based on the variations in three restriction sites (two located in the unique central part and one located in the prDNA) after digestion with *Eco*R1, *Bam*H1 and *Hind*III. In addition to the variations in the number of restriction sites, fragment size variations are also observed.

In agreement with the restriction enzyme studies, the phylogenetic analysis demonstrated that there is a high genetic variability among the field strains. The phylogenetic groupings identified were strongly supported by the clades depicted in Fig. 5, which show probabilities above 98%. However, this grouping scheme should be confirmed by the analysis of other molecular markers. It has been shown for other herpesviruses that the evolutionary histories of different genomic regions can be complex, and, thus, that viral classifications must be grounded in the analyses of several molecular markers (Shin *et al.*, 2010; Nicholas *et al.*, 1998).

These results indicate that there is a high degree of genomic variation among the different BoHV-4 field isolates. It is important to consider that differences in restriction enzyme profiling have also been observed by other authors (Yamamoto *et al.*, 2000; Izumi

283 *et al.*, 2006). Nevertheless, in this study, the differences were more remarkable. As these
284 differences in the restriction enzyme patterns are likely due to single-nucleotide
285 polymorphisms (SNPs) that result in the loss or gain of restriction endonuclease sites, it can
286 be expected that other genomic regions could present similar levels of SNPs, which
287 emphasizes the importance of performing deeper genomic analyses.

288 These results suggest a complex epidemiological background for BoHV-4 strains,
289 possibly influenced by independent events of genetic drift. The involvement of this genetic
290 variability on detection and pathogenesis of BoHV-4 remains to be investigated.

291 The existence of different viral genotypes suggests the possibility of some
292 association between genetic variations and particular pathogenic potentials, specially for
293 the two strains that didn't cluster into any of the Genotype 1, 2 or 3, which, furthermore,
294 correspond to abortions in which no other pathogen was identified. Sequencing of a larger
295 number of field isolates, together with adequate recording of the associated clinical and
296 epidemiological data, will contribute to determine whether the viral genetic background can
297 influence the clinical outcome of infection and to further characterize the degree of
298 genomic divergence among the Argentinean BoHV-4 isolates.

299 Information obtained by restriction pattern length polymorphism and phylogenetic
300 analysis provides a basis for the characterization of new BoHV-4 isolates. Molecular
301 investigation has proved to be extremely helpful in addressing these matters for others
302 animal and human herpesviruses.

303 In this study, we sequenced only the TK fragment of the viral genome, which hardly
304 could be a determinant of viral pathogenesis. Thus, further studies of the molecular
305 characteristics of BoHV-4 field strains need to be carried out to untangle the genetic basis

of viral pathogenesis. In particular, it would be especially interesting to evaluate field isolates from cows with clinical conditions different from post-partum metritis.

## Conclusion

Overall, this work demonstrates that there is a high variability among the BoHV-4 strains isolated from vaginal discharges of aborted cows from Argentina. Although in many cases other pathogens were identified, it is clear that BoHV-4 is an agent which has the potential to be responsible for reproductive diseases in cattle.

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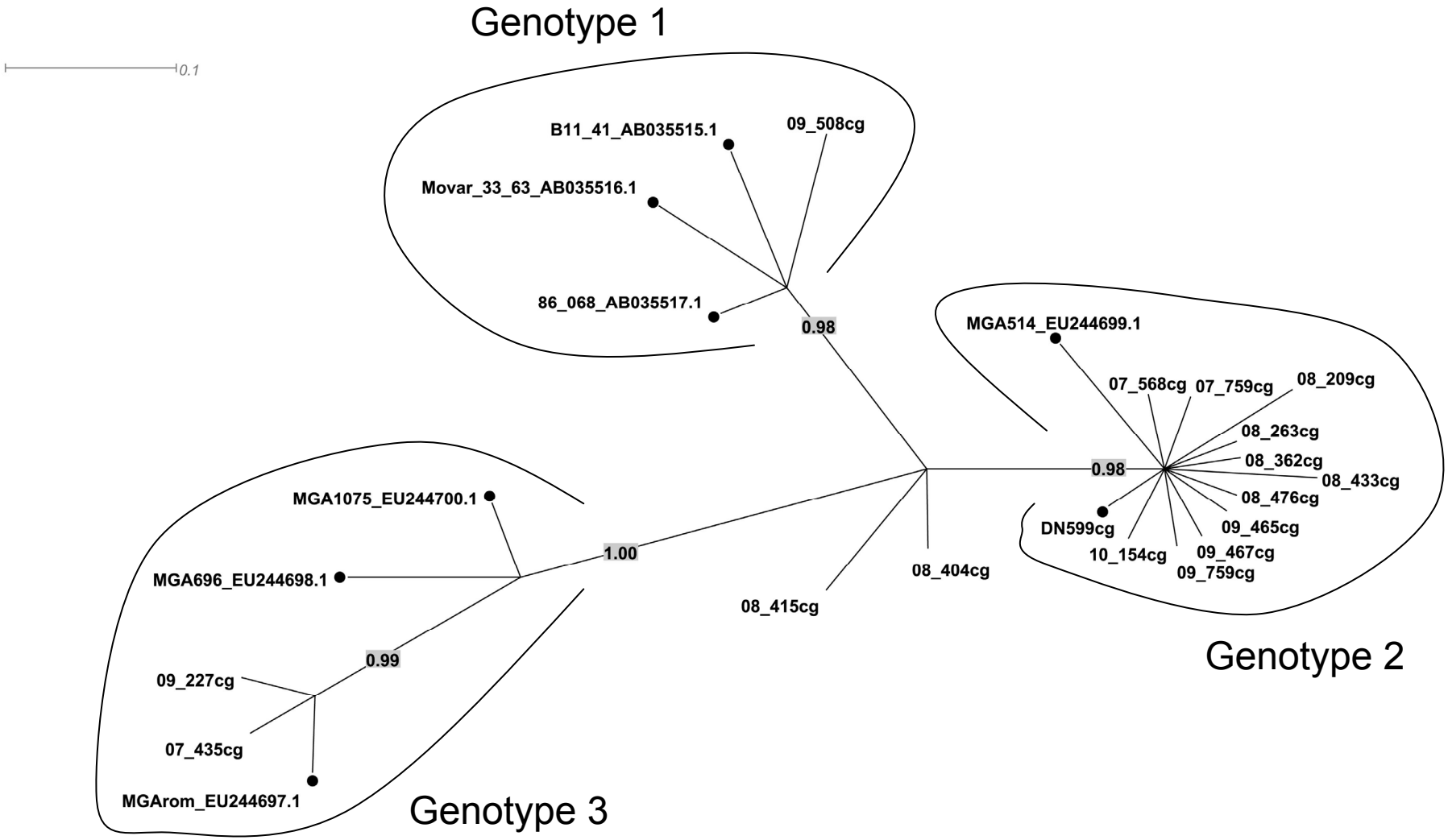
**Figure**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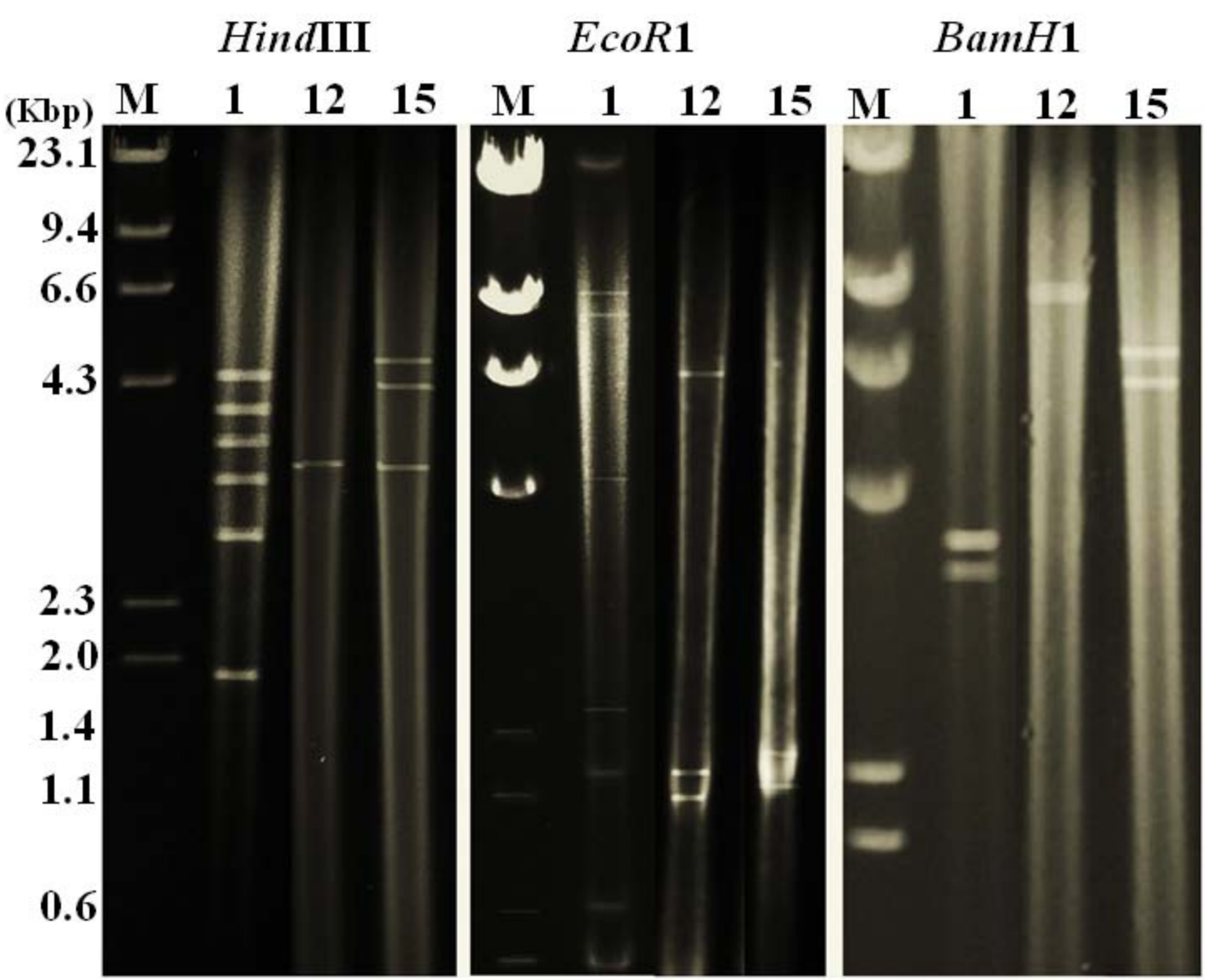
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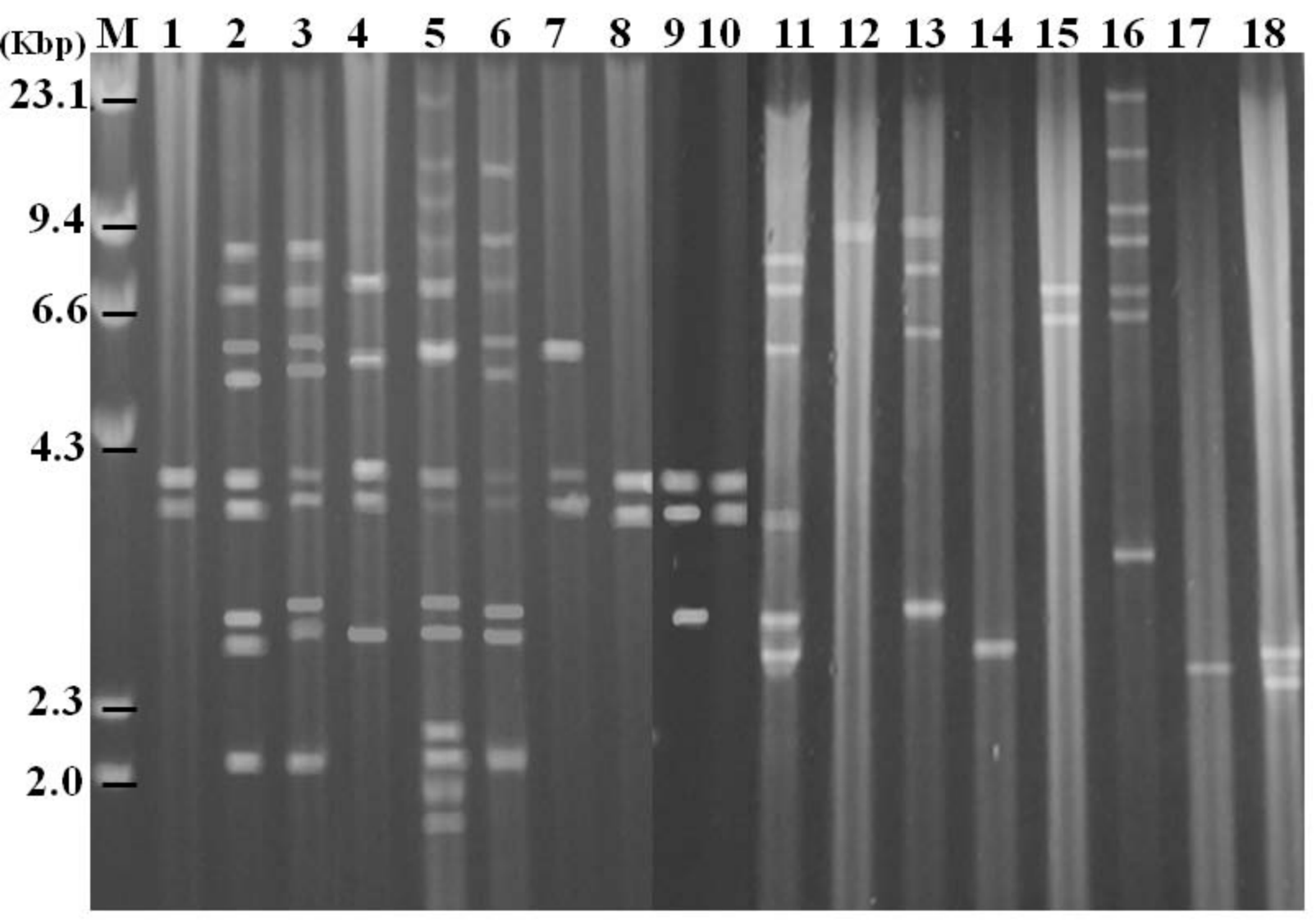
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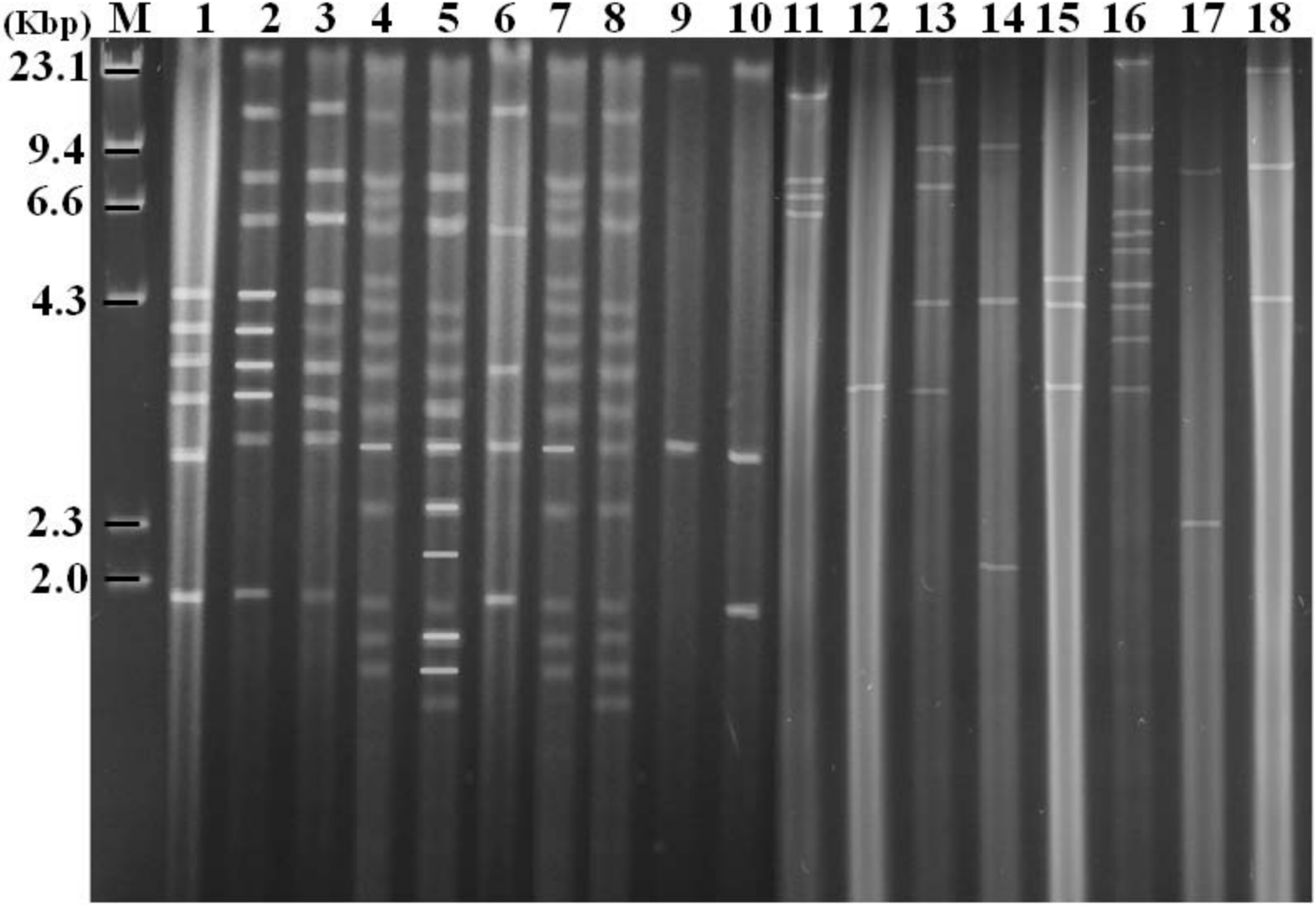
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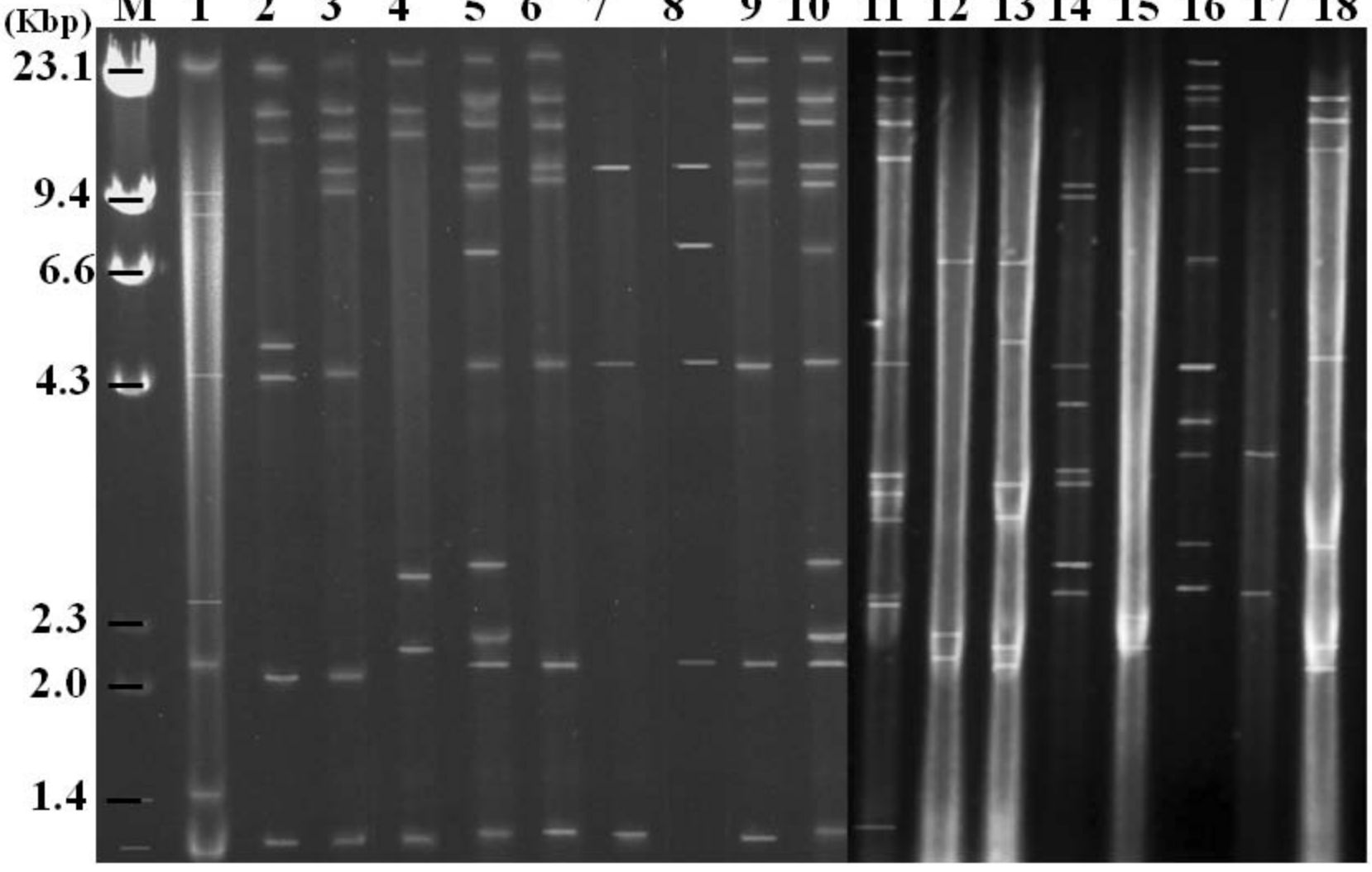
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| N° | Isolates | Location | Comments |
|---|---|---|---|
| 1 | Reference Strain DN599- like Group | USA | |
| 2 | 08/209 | Carmen de Areco | Aborted cows were seropositive to *Neospora caninum.* Non aborted cows were seronegative*;* high antibody titers to BVDV are evident. Serum antibody titers to some *Leptospira* spp. serovars are also present. |
| 3 | 08/476 | Pehuajo | Negative to regular agents associated with abortion |
| 4 | 07/568 | Trenquen Lauquen | Seroconversion to BVDV and animals seropositive to *N. caninum* were detected. *Histophillus somni* was isolated from the vaginal discharge; titers 1/800 to *Leptospira interrogans* serovar Hardjo |
| 5 | 07/435 | Vedia | Abortion-causing pathogens were not identified. Circulating antibodies to some *Leptospira* serovars were detected. |
| 6 | 08/433 | Pehuajo | Negative to regular agents associated with abortion |
| 7 | 08/263 | Gral. Pueyrredón | Isolation of *Arcanobacterium pyogenes* |
| 8 | 08/330 | | |
| 9 | 09/759 (6007) | San Miguel del Monte | Abortion attributed to multiple infectious causes. Seropositive to *N. caninum (*Titer: 1/800). Non citopathic BVDV was detected in vaginal discharges. Fescue-grass parasited with a high percentage of the endophyte fungi *Neotyphodium* |
| 10 | 07/759 (7850) | San Miguel del Monte | Abortion attributed to multiple infectious causes. Seropositive to *N. caninum (*Titer: 1/800). Non citopathic BVDV was detected in vaginal discharges. Fescue-grass parasited with a high percentage of the endophyte fungi *Neotyphodium* |
| 11 | 10/154 | Venado Tuerto | Antibody titer to *Leptospira interrogans* serov. Hardjo: 1/200. *Arcanobacterium pyogenes* detected in the vaginal discharge. Presence of neutralizing antibodies to BVDV and BoHV-1 and 5 |
| 12 | 08/404 | Balcarce | Negative to regular agents associated with abortion |
| 13 | 08/362 | Gral. Belgrano | |
| 14 | 09/465 | Maipú | Seropositive to *N. caninum* |
| 15 | 08/415 (950) | Bahía Blanca | Negative to regular agents associated with abortion |
| 16 | 09/508 | Córdoba | Isolation of *Arcanobacterium pyogenes* |
| 17 | 09/227 | Gral. Alvear | Negative to regular agents associated with abortion |
| 18 | 08/467 | 9 de Julio | Negative to regular agents associated with abortion |