Analysis of the PLD gene of Corynebacterium pseudotuberculosis

Diehl, Maya Stéphanie

Institut für Veterinärbakteriologie
der Vetsuisse-Fakultät Universität Zürich
Direktor: Prof. Dr. Max M. Wittenbrink

Analysis of the PLD gene of *Corynebacterium pseudotuberculosis*

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Maya Stéphanie Diehl

Tierärztin

von Degersheim SG, CH

genehmigt auf Antrag von

Prof. Dr. Max M. Wittenbrink, Referent

PD Dr. Adrian B. Hehl, Korreferent

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To my beloved mother

Thanks for all you're giving me

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1. Summary

Corynebacterium pseudotuberculosis (Cp) is the causative agent of caseous lymphadenitis (CL), disease affecting small ruminant worldwide. Although a great homogeneity both phenotypically and genetically exists among the *Cp* strains, the nitrate reduction capability allows the subdivision of the species in two biovar *ovis* and *equi*. Our study investigated the homology of *Cp* using phospholipase D (PLD) gene, an immunodominant antigen playing a major role in the virulence of CL.

Besides 6 other corynebacterial reference strains and *Arcanobacterium haemolyticum*, two reference and 23 randomly selected swiss field strains of Cp were screened for the presence of PLD gene using a specific PLD PCR. The RFLP analysis with Alu I showed an identical pattern developed by all *Cp* strains tested. A southern blot analysis revealed a difference in the number of PLD gene copies between biovar. PLD gene from 8 field strains and the 2 *Cp* reference strains was cloned and sequenced, and the resulting nucleotide and amino acid sequences were compared: genetic divergences between biovar and within biovar *ovis* were found, revealing the existence of 3 different structures of the PLD protein. Further studies are necessary to investigate whether these results could e.g. explain differences observed in CL clinical symptoms, pathology or host preference.

2. Zusammenfassung

Corynebacterium pseudotuberculosis (*Cp*) ist der Erreger der Pseudotuberkulose, einer Krankheit, die weltweit vor allem kleine Wiederkäuer befällt. Obgleich eine grosse Homogenität sowohl phenotypisch wie genetisch unter den *Cp* Stämmen besteht, erlaubt die Fähigkeit der Nitratreduktion die Unterteilung der Spezies in zwei Biovare, i.e. Biovar *ovis* und Biovar *equi*. Ziel der vorliegenden Arbeit war, ob das Gen für die Phospholipase D (PLD), der wichtigste Virulenzfaktor von *Cp,* geeignet ist, um die phänotypische Homogenität innerhalb der Spezies nachzuvollziehen.

Insgesamt wurden Vertreter von sechs Spezies der Corynebakterien-Familie, *Arcanobacterium haemolyticum*, sowie zwei Referenz- und 23 Feldisolate von *Cp* auf das Vorhandensein des PLD Gens in der PCR geprüft. Die RFLP Analyse der PCR Produkte mit Alu I zeigte für alle *Cp*-Stämme ein identisches Bandmuster. Mittels Southern blot Analyse zeigte sich, dass *Cp* Biovar ovis fünf Kopien des PLD Gens trägt, während im Referenzstamm des Biovar equi sechs Kopien des PLD Gens nachweisbar waren. Das PLD Gen-Amplifikat von acht Feldstämmen und von den beiden *Cp* Referenzstämmen wurde kloniert und sequenziert. Die Analyse der Nukleotidsequenzen und die 2D-Analysen der daraus abgeleiteten Aminosäuresequenzen ergaben, dass die Struktur des PLD-Gens innerhalb des Biovar *ovis* und zwischen den beiden Biovaren *ovis* und *equi* deutliche Unterschiede aufweisen. Die Resultate können als Hinweis bewertet werden, dass es innerhalb des hierzulande relevanten Pathogens *Cp* Biovar *ovis* genetisch unterschiedliche Vertreter gibt. Ob diese Unterschiede genutzt werden können etwa als erregerseitiger Marker z.B für die unterschiedlichen klinischen Verlaufsformen der Pseudotuberkulose, muss in weiteren Studien geklärt werden.

3. Introduction

Caseous lymphadenitis is a world-wide disease of significant economic importance affecting sheep and goats. The disease is characterized by abscess formation in superficial and/or internal lymphnodes and organs (Batey, 1986; Brown et al., 1987). It results in a loss of milk, meat and wool production and in the condemnation of carcasses presenting internal abscesses. The causative agent is the gram-positive rod-shaped bacterium *Corynebacterium pseudotuberculosis* (below referred to as *Cp*). *Cp* is also known as a causative agent of an ulcerative lymphangitis or subacute abscesses in horses (Pratt et al., 2005). Man as well as animal species such as cattle may occasionally be affected (Peel et al., 1996; Yeruham et al., 1997; Anderson et al., 2004).

Cp can be identified phenotypically on the basis of cultural and biochemical characteristics (Muckle et al., 1982) although some variations have been recorded within the strains studied so far. These variations appeared mainly due to differences in the methods used to characterize *Cp* (Songer et al., 1988). The species was subdivided into two biovars using the nitrate reduction capability (Biberstein et al., 1971): *Cp* biovar *ovis*, nitrate-negative, causing caseous lymphadenitis in small ruminants, and *Cp* biovar *equi*, nitrate-positive, principally isolated from horses. The genetic differences obtained using restriction fragment length polymorphism RFLP (Songer et al., 1988; Sutherland et al., 1996; Costa et al., 1998) methods correlate with the subdivision in 2 biovars.

Although variations have been found within each of the biovars, the comparison of strains isolated from different geographical locations demonstrates great homogeneity both phenotypically and genetically (Costa et al., 1998; Connor et al., 2000). There was no difference within the biovar *ovis* to explain the variability of clinical pathology and distribution of the abscesses in sheep and goats (Sutherland et al., 1993).

Cp is characterized by the presence of a phospholipase D (PLD) which plays a vital role in the pathogenesis of ovine and caprine caseous lymphadenitis (Barksdale et al., 1981). Bacterial PLDs including the *Cp*-PLD promote the hydrolysis of ester bonds in the membrane-bound phospholipid sphingomyelin of mammalian cells (Tambourgi et al., 2002). The PLD from *Cp* biovar *ovis* has been established as the essential virulence factor which hydrolyses the sphingomyelin component of

erythrocyte and vascular endothelial membranes and contributes to the spread of bacteria from the initial site of infection (Bernheimer et al., 1980; Tachedjian et al., 1995; McNamara et al., 1995). Moreover, PLD activates neutrophiles and complement (Tambourgi et al., 2002; Yozwiak and Songer, 1993). PLD has been detected so far in all *Cp* strains studied (Brown et al., 1987; Songer et al., 1988). The strains which produced no PLD have apparently been misclassified as *Cp* (Barksdale et al., 1981). PLD is an immunodominant antigen and stimulates a humoral and cellular immune response in affected small ruminants (Muckle et al., 1992; Menzies et al., 2004). PLD is therefore used for serodiagnosis of *Cp*-infected animals as well as a vaccine (Doty et al., 1964; Burrell, 1980 (3 publications); Sutherland et al., 1989; ter Laak et al., 1992; Dercksen et al., 2000) and vaccine production (Eggleton et al., 1991; Simmons et al., 1998; Hodgson et al., 1999).

Because of the key role of PLD in the pathogenesis of caseous lymphadenitis, the encoding gene could probably represent a suitable target for investigating genetically the pathogenic divergence of *Cp* strains. In fact, differences in the structure of PLD might possibly explain the difference observed in virulence and localization of lesions between sheep and goats. Since 1999, more than 100 *Cp* strains from Swiss sheep and goats suffering from caseous lymphadenitis have been isolated at our institute.

In the present study, a panel of 23 *Cp* field strains, two *Cp* reference strains, six related *Corynebacterium spp*. and a reference strain of *Arcanobacterium haemolyticum* were subjected to a molecular analysis of the PLD gene.

4. Material and Methods

4.1. Bacterial strains

A total of 23 field isolates of *Cp* biovar *ovis* strains were grown from sheep (n=5) and goats (n=18) with cutaneous suppurative conditions strongly indicative for clinical pseudotuberculosis. Samples were collected from different regions in Switzerland by the Swiss Extension and Health Service for Small Ruminants (Herzogenbuchsee, CH). Isolates were identified by standard microbiological techniques (Quinn et al., 1994) including the commercially available Api Coryne test (bioMérieux, Marcy l'Etoile, France). The following reference strains were used as comparators throughout the biochemical characterization and the analysis of the PLD gene: *Cp* biovar *ovis* DSM 20689, *Cp* biovar *equi* DSM 7177, *Corynebacterium ulcerans* DSM 46325, *Corynebacterium diphtheriae* DSM 44123, *Corynebacterium kutscheri* DSM 20755, *Corynebacterium bovis* DSM 20582, *Corynebacterium cystitidis* DSM 20524, *Corynebacterium renale* DSM 20688, *Arcanobacterium* (*A*.) *haemolyticum* DSM 20595. Cultures were preserved by inoculating a Protect vial (Technical Service Consultants Ltd., Heywood, GB) according to the manufacturer's instructions and were stored at -70°C. Bacterial strains were grown on Columbia blood agar (Oxoid, Basel, Switzerland) in a 5% CO_2 incubator at 37°C for 48 h, and were passaged no more than four times from initial extraction of genomic DNA to testing by PCR.

4.2. Purification of genomic DNA

For DNA extraction, each bacterial strain was grown in 5 ml Luria-Bertani broth (LB broth) for 36-48 h at 37°C and aerobic atmosphere under constant agitation at 120 rpm. Total DNA was obtained from corynebacterial cultures by a direct lysis method. 400 µl of cultures or controls were mixed with 45 µl lysis buffer (100 mM Tris, 10 mM EDTA, 1 M NaCl, pH 7.4; 10% sodium dodecyl sulfate). Proteinase K was added to a final concentration of 0.1 mg/ml. Samples were incubated at 56°C for 2 h and then heated at 100°C for 10 min in order to inactivate the proteinase K. DNA extraction was accomplished three times with an equal volume of a mixture of phenol-chloroform-isoamyl alcohol (25:24:1) according to a standard protocol (Sambrook et al., 1989). DNA was precipitated by adding 0.1

volume of 3 M sodium acetate and 2 volumes of chilled absolute ethanol (-70°C, 30 min) and pelleted by (15000 x g, 4°C, 30 min). DNA pellets were rinsed in 70% ethanol, air dried, and dissolved in 20 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). DNA concentrations were determined spectrophotometrically at 260 nm (GeneQuant, Pharmacia).

4.3. PCR amplification of the PLD gene

The PCR specific primers were designed in order to amplify nearly the entire *Cp* PLD gene by using a primer selection software (OLIGO Primer Analysis Software version 4.0, MBI, USA) and correspond to the positions 1 to 26 (primer 1, 5'-ATG AGG GAG AAA (GT)TT G(CT)T TTA TT(AC) TT-3') and 907 to 923 (primer 2, TCA CCA CGG GTT ATC GC-3') in the *Cp* PLD gene (GenBank accession number L16586). Primers were synthesized by MWG Biotech (Ebersberg, Germany). The PCR reaction mixture consisted of 200 nM of each primer, 200 µM (each) dATP, dCTP, dGTP, and dTTP; 10.0 mM Tris/HCl, pH 8.3; 2.5 mM $MgCl_2$; 50.0 mM KCl, 2.5 U of *Taq* DNA polymerase (Boehringer, Mannheim, Germany), and 1.0 µl of template DNA in a total volume of 50 µl. Thermal cycling conditions were as follows: initial denaturation (2 min, 94°C) followed by 30 cycles of denaturation (30 sec, 94°C), annealing (30 sec, 52°C), extension (1 min, 72°C). A terminal extension step was carried out for 7 min at 72°C. All PCR reactions were performed in a DNA thermal cycler 2400 (Perkin-Elmer, Weiterstadt, Germany). Included in each PCR run were positive and negative extraction controls. To ensure that no contaminating DNA would give positive results, negative amplification controls (2 µl of the sterile water used to prepare the PCR mix) were included in every PCR run. Overall, at least three replicates of each PCR were performed independently in order to confirm reproducibility. PCR products were separated on 1% agarose gels (Agarose NEEO, Roth, Karlsruhe, Germany) stained with ethidium bromide. Amplification products were sized under UV light with reference to DNA 1 Kb molecular size marker lanes (Invitrogen, Breda, Netherlands) by using a computer-aided bio-image system (BioProfil 3.1, LTF, Wasserburg, Germany).

4.4. RFLP analysis

Amplification products from the PLD-PCR were purified by using the QIAquick purification kit (Qiagen, Hilden, Germany). Restriction endonuclease *Alu* I (Boehringer, Mannheim, Germany) was used to cleave PCR products according to the manufacturer‘s recommendations. 40 µl of each PLD-PCR product was cleaved with 10 units *Alu* I at 37°C overnight. Following digestion, the DNA fragments were precipitated with 3 M Na-acetate and absolute ethanol to concentrate the sample, and dissolved in 10 µl TE buffer. Restriction digests (10 µl) were analyzed by gel electrophoresis for 2 h at 50 V on a 2.0% agarose gel (Small DNA Agarose, Biozym, Oldendorf, Germany) containing 0.5 µg per ml ethidium bromide. A 100 bp DNA ladder was used as an external DNA molecular size marker (Invitrogen). DNA fragments were sized under UV light with reference to the 100 bp DNA molecular size marker lanes by using a computer-aided bio-image system (BioProfil 3.1). Reproducibility of restriction endonuclease analysis was confirmed by reanalyzing PCR amplification products of *Cp* reference strains.

4.5. DNA sequencing

PLD-PCR amplification products were cloned into the plasmid vector pCR2.1 using the kit (Invitrogen) according to the manufacturer’s instructions. The plasmid DNA of each clone was then purified with the Qiagen Plasmid Kit (Qiagen, Hilden, Germany). Sequencing was performed by MWG Biotech (Ebersberg, Germany). Nucleotide sequences were analysed and aligned to each other by using HUSAR (Heidelberg Unix Sequence Analysis Resources). Nucleotide and deduced amino acid sequences were compared with data bank entries of the PLD of other *Corynebacteriaceae* strains accessioned in the EMBL and GenBank database.

4.6. Southern blot analysis

The 923-bp PLD-PCR product amplified from the reference strains *Cp* biovar *ovis* DSM 20689 and *Cp* biovar *equi* DSM 7177 were used as DNA probes (probe *Cp ovis* and probe *Cp equi*). 100-500 ng of purified DNA (QiaEXII, Qiagen, Hilden, Germany) were labelled with digoxigenin-11-dUTP by a random oligonucleotide primer method in accordance with the manufacturer’s instructions (Boehringer, Mannheim, Germany).

DNA extraction from the investigated field isolates as well as from reference strains was performed as described above. 10-12 µg of genomic *Cp* DNA was cleaved with 10 units *Bam H*I at 37°C overnight. Following digestion, the DNA fragments were precipitated with 3 M Na-acetate and absolute ethanol, and redissolved in 20 µl bidistilled water. DNA fragments were separated electrophoretically on 0.8 % agarose gel at a constant voltage of 30 V during 24 h. Agarose gels containing the *Bam H*I fragments were washed twice in 0.25 N HCl (5 min) followed by three washings in bidistilled water (5 min). Afterwards, the gels were denatured for 30 min under gentle agitation using 0.5 N NaOH and 1.5 M NaCl. Agarose gels were then blotted onto a nylon membrane (Hybond-N, Amersham, Freiburg) by alkaline capillary blotting in accordance to a standard procedure (Sambrook and Russell, 2001). After rehydrating in 5 x SSC, the membranes were incubated with 8 ml DIG-Easy-Hyb-solution (Roche) for 30 min at room temperature under gentle agitation. Unspecific binding sites were blocked by incubating the membranes in 100 ml 1% blocking reagent in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) for 1 h under gentle agitation at ambient temperature. The hybridization reaction was performed overnight at ambient temperature. After hybridization, the membranes were washed at ambient temperature twice for 5 min in 2x SSC-0.1% SDS followed by two subsequent washings at 62°C for 20 min in 0.1 x SSC-0.1% SDS. Immunodetection was performed with antidigoxigenin antibodies conjugated with alkaline phosphatase, and the immune complexes were visualized by using a colorimetric detection system according to the manufacturer's instructions (Boehringer, Mannheim, Germany). Colour development was clearly visible between 15 min and 1 h after start of reaction.

5. Results

5.1. PCR-RFLP analysis of the *C. pseudotuberculosis* PLD gene

By using DNA from the reference strains of *Cp* biovar *ovis* and *Cp* biovar *equi* as a template, single DNA-fragments of the expected size of 900 bp were obtained in the PLD-PCR. The identity of these PCR products was confirmed by nucleotide sequence analysis of purified DNA clones of both PCR amplicons. Nucleotide sequences revealed 99.9% identities with the *Cp* PLD gene (GenBank Acc.-No. L16586).

PCR amplification products of the expected size were also obtained from DNA extracted from 23 field isolates of *Cp* biovar *ovis*. Nucleotide sequences of PCR products from ten randomly selected *Cp* field isolates were also 99.9 to 100% identical with the reference sequence (Acc.-No. L16586). PCR products of the expected size were neither amplifyable from the reference strains of five heterologous corynebacterial species known not to have PLD nor from PLD carrying *C. ulcerans* or the *A. haemolyticum* reference strain, respectively.

After digestion of the PCR amplicons with *Alu* I, the *Cp* reference strains as well as the 23 *Cp* field isolates revealed identical homogeneous RFLP patterns consisting of four bands of 230, 220, 195 and 171 bp respectively (Fig.1).

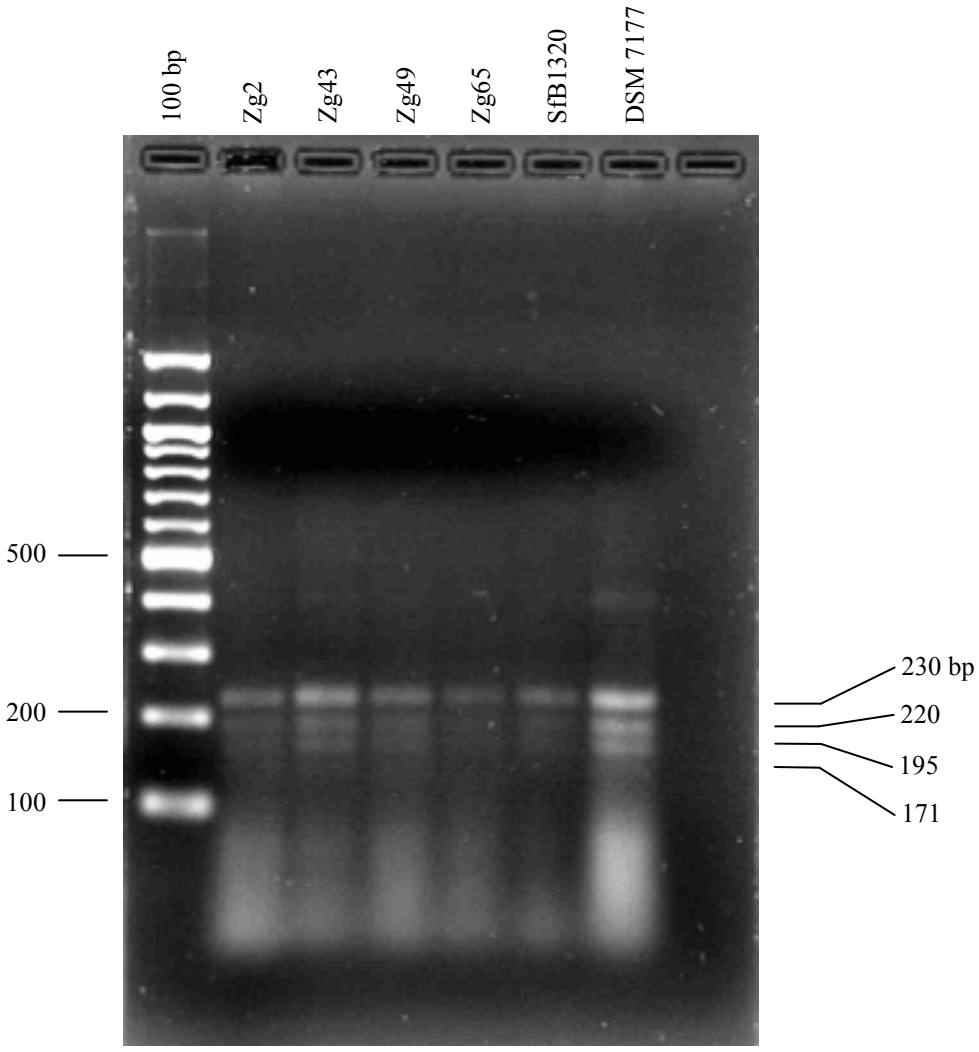

Fig.1 RFLP pattern of four bands obtained after digestion of the PLD-PCR amplicons with Alu I. Lane 1 represents the 100 bp ladder. Lanes 2 to 6 correspond to the field strains *Cp* biovar *ovis* Zg2, Zg43, Zg49, Zg65 and SfB1320 respectively. Lane 7 contains the *C. pseudotuberculosis* biovar *equi* reference strain.

5.2. DNA sequencing of PLD gene

The PLD-PCR amplification products from both *Cp* reference strains as well as from a sample selected at random of eight *Cp* field isolates were cloned and sequenced (Table 1).

Id number	biovar	RFLP pattern	PLD gene copies	2D model
DSM 20689	*Cp* biovar *ovis*	4	5	A
DSM 7177	*Cp* biovar *equi*	4	6	B
Swiss strains	*Cp* biovar *ovis*			
Sf41	″	4	5	A
Sf63	″	4	5	A
Sf67	″	4	5	A
Sf111	″	4	5	–
SfB1320	″	4	5	A
Zg2	″	4	5	–
Zg3	″	4	5	–
Zg22	″	4	5	A
Zg27	″	4	5	A
Zg29	″	4	5	–
Zg32	″	4	5	–
Zg43	″	4	5	A
Zg44	″	4	5	–
Zg49	″	4	5	–
Zg51	″	4	5	–
Zg52	″	4	5	–
Zg54	″	4	5	C
Zg58	″	4	5	–
Zg61	″	4	5	–
Zg65	″	4	5	–
Zg79	″	4	5	–
Zg109	″	4	5	–
Zg110	″	4	5	–
DSM 46325	*C. ulcerans*	–	–	–
DSM 20595	*A. haemolyticum*	–	–	–
DSM 20524	*C. cystitidis*	–	–	–
DSM 20582	*C. bovis*	–	–	–
DSM 20688	*C. renale*	–	–	–
DSM 20755	*C. kutscheri*	–	–	–
DSM 44123	*C. diphteriae*	–	–	–

Table 1. Results obtained studying *C. pseudotuberculosis*, corynebacterial reference strains and *A. haemolyticum* using PCR, RFLP, DNA sequencing and Southern blot analysis.

Comparing among the 10 strains, the analysis of nucleotide sequences revealed the presence of a variable number of point mutations. Few of them ended in a change of the resulting amino acid sequence, revealing 3 different 2D structures of PLD protein, namely A to C (Fig.2.a, 2.b, 2.c). Model A brings together 7 field strains and the reference biovar *ovis*; model B is constituted from biovar *equi* reference strain; model C contains the field strain Zg 54.

Fig. 2.a

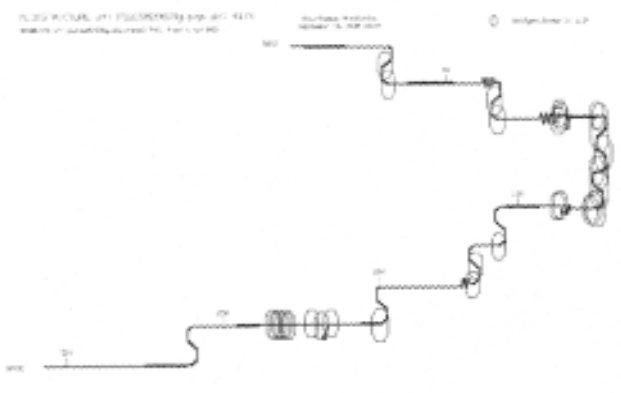

Fig. 2.b

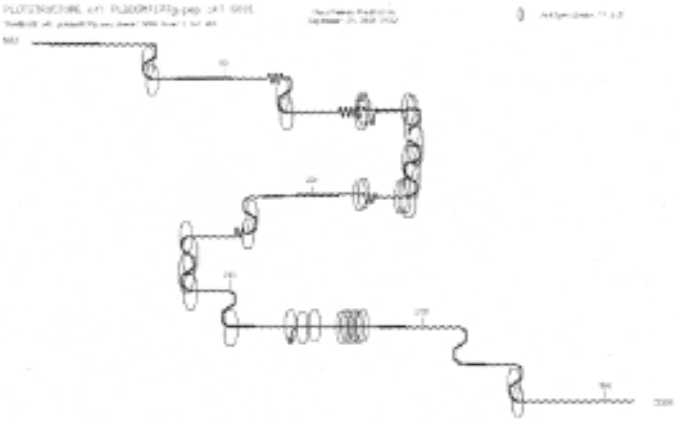

Fig. 2.c

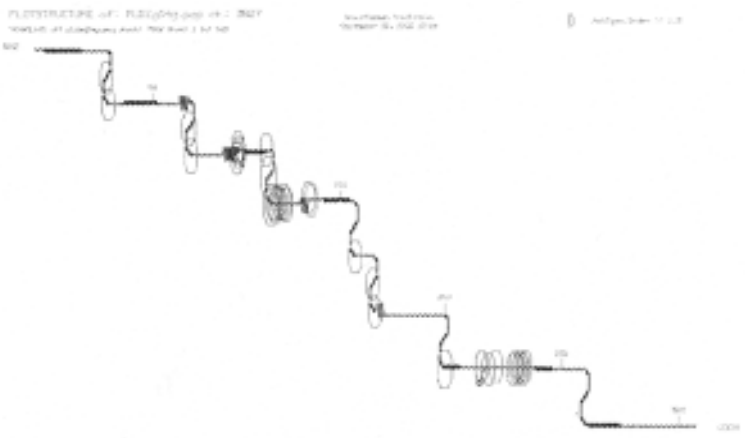

Fig.2 The 3 PLD protein-2D models obtained by comparing 10 gene sequences of *Cp* using HUSAR. a) *Cp* biovar *ovis* DSM 20689 and 7 *Cp* swiss field strains b) *Cp* biovar *equi* DSM 7177 c) *Cp* Zg54.

5.3. Southern blot analysis

All of the biovar *ovis* strains tested twice presented the same pattern of five bands from 10.8, 10.6, 9.64 and 8.43 to 7.1 Kb (Fig.3) respectively, while the biovar *equi* showed a sixth specific band of 3.97 Kb. All these bands were isolated and the presence of PLD gene was verified using PLD-PCR. No reaction was detected testing either *C. bovis* or *A. haemolyticum* and *C. ulcerans* as controls (Fig.4).

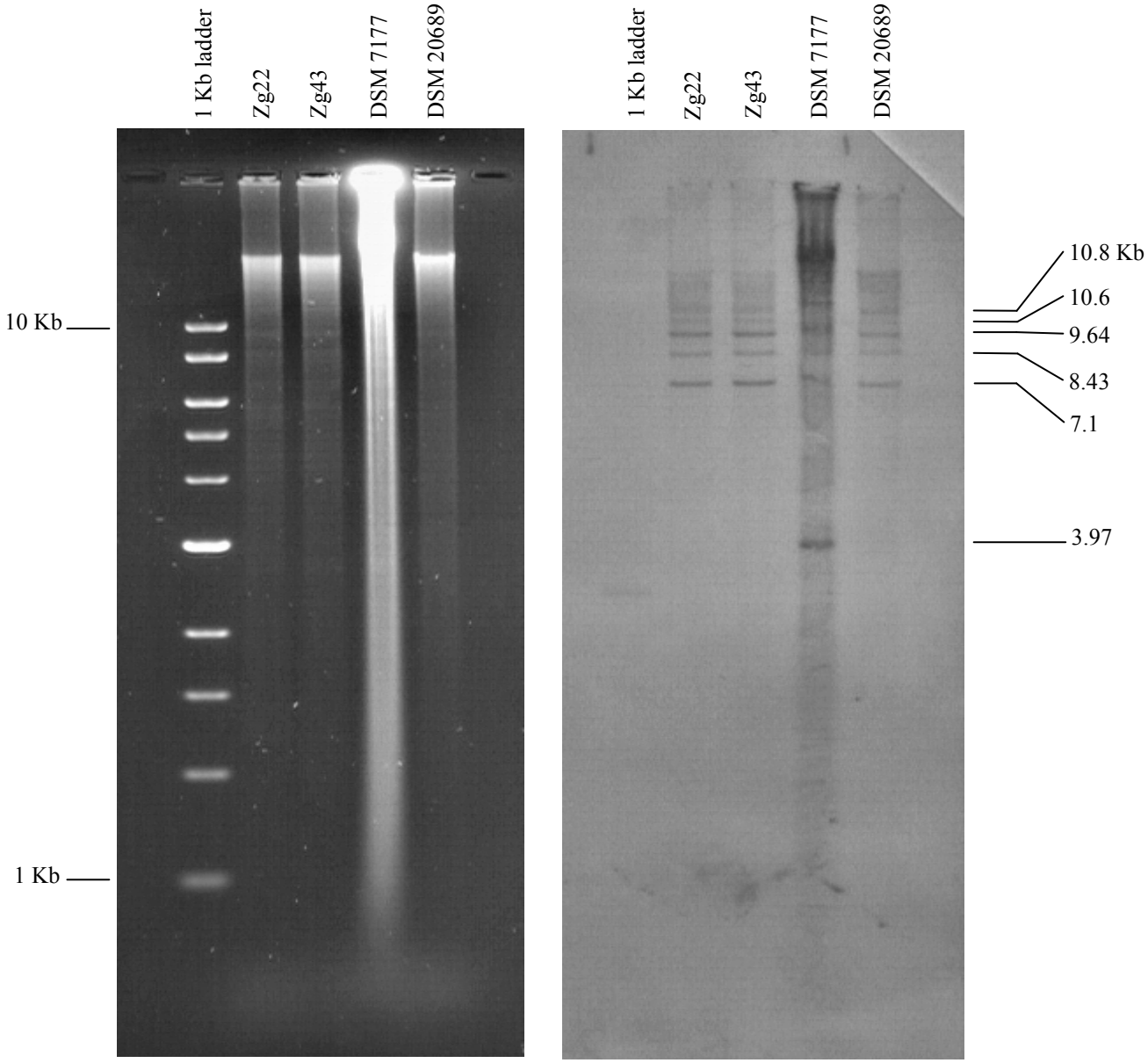

Fig.3 Southern blot analysis reveals a same pattern of 5 bands by 2 *Cp* field strains and the reference strain *Cp* biovar *ovis* (lanes 2, 3 and 5). *Cp* biovar *equi* (lane 4) differs from the others with a sixth band of 3.97 Kb.

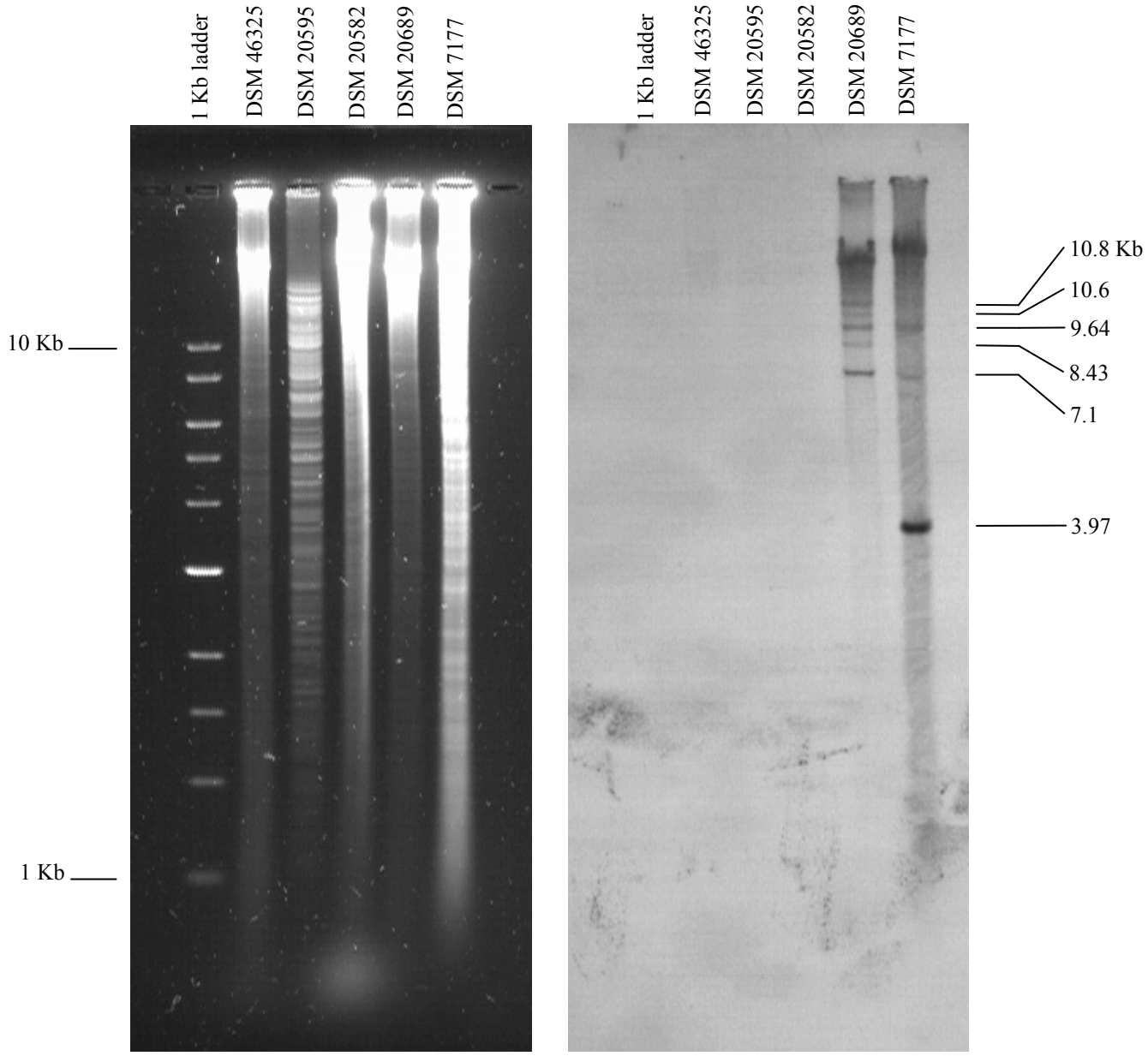

Fig.4 Southern blot analysis presents the pattern of 5, respectively 6 bands obtained by *C. pseudotuberculosis* biovar *ovis* and *C. pseudotuberculosis* biovar *equi* (lane 5 and 6). None of the PLD gene of *C. ulcerans* and *A. haemolyticum* (lanes 2 and 3) was detected, which confirms the specificity of our probe. *C. bovis* (lane 4) shows no reaction in keeping with the absence of PLD gene.

6. Discussion

This study intended to investigate the differences existing between sheep and goat in the clinic and the pathology of CL, using the gene of the PLD, one of its major factors of virulence.

The PLD gene is present not only in *C. pseudotuberculosis* but also in other species such as *C. ulcerans*, *A. haemolyticum* and the spider *Loxosceles intermedia* (Tambourgi et al., 2002). The studies already done on PLD showed a homology between PLD gene of *C. pseudotuberculosis*, *A. haemolyticum* and *C. ulcerans* from 64 % (Cuevas et al., 1993; MacNamara et al., 1995) and 80 % (MacNamara et al., 1995) respectively, and from 98 % between the biovar *ovis* and *equi* (MacNamara et al., 1995). One complete sequence of the PLD gene of *C. pseudotuberculosis* and its resulting amino acid chain was published by Hodgson (Hodgson et al., 1990). The PLD gene sequence belonging to *C. ulcerans* and *A. haemolyticum* are also known (MacNamara et al., 1995).

For the amplification of the *C. pseudotuberculosis* PLD gene, specific primers were designed by analyzing already known PLD gene sequence. The amplification obtained for all *Cp* strains tested demonstrated the constant presence of a complete and potentially active PLD gene. The fact that none of the species employed as control, inclusive *A. haemolyticum* and *C. ulcerans*, were amplified, seems to prove the specificity of the primers used for our PCR, basic work for the next steps of the study.

Different methods have been used until now to attempt to find genetic divergences between strains of *C. pseudotuberculosis*. This species shows phenotypically a great homogeneity in cultural and biochemical properties. Only the ability to reduce nitrates can divide the species in two biovar. Several studies made at the genomic level revealed divergences existing principally between biovar *ovis* and *equi*, by using enzymatic restriction methods such as REA (Songer et al., 1988) and RFLP (Sutherland et al., 1993; Sutherland et al., 1996; Costa et al., 1998). Moreover other studies pointed out that differences within biovar levels are less common (Costa et al., 1998; Connor et al., 2000).

However, Songer (Songer et al., 1990) demonstrated by Southern blotting method a difference between biovar *ovis* and *equi*. They hybridized a PLD probe cloned in *Escherichia coli* on DNA digested with Bam HI. We used a similar method

with a probe obtained by amplification of the PLD gene with our specific primers. Our results showed for all strains tested that there is not only a genetic divergence between biovar, but also a greater number of fragments which hybridized positively in the blott. This seems to demonstrate the presence of a higher number of PLD gene copies in the genome of *C. pseudotuberculosis* from the strains tested as reported by Songer (Songer et al., 1990) and Hodgson (Hodgson et al., 1990).

We found also others differences at the biovar level by sequencing the PLD gene. In our study, 8 field strains of *C. pseudotuberculosis* biovar *ovis* and two *Cp* reference strains were cloned and sequenced. The comparison of the nucleotide sequence demonstrated a number of point mutations, resulting sometimes in a modification of the amino acid sequence. The predictive 2D analysis allows to point out the existence of 3 different structures of the PLD protein. This reveals the presence of a genetic divergence at the level of PLD gene between biovar *ovis* and *equi* on one hand, and between strains of biovar *ovis* on the other hand.

As emphasized by Songer (Songer et al., 1990), the presence of a different number of PLD gene copies between biovar as well as the genetic divergence in the sequence of nucleotides and amino acids between biovar and within the biovar *ovis* could play a role in the virulence factor from the PLD. All these results could explain the differences observed in CL clinic, pathology and host preference.

To verify this hypothesis, more detailed analyses are required to investigate these observed genetic divergences and their implication in the pathogenesis and the immunity developed by the host.

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Lebenslauf

Name	Maya Stéphanie, Diehl
Geburtsdatum	11. 02. 1974
Geburtsort	Levallois-Perret, France
Nationalität	Schweiz
Heimatort	Degersheim SG

1980-1985	Primarschule, Colombier / NE
1985-1989	Sekundarschule lateinische Sektion, Colombier / NE
1989-1992	Maturitätsschule, lycée Denis-de-Rougemont, Neuchâtel
1992	Eidgenössische Maturität Typ C, Neuchâtel

1992-1998	Studium der Biologie, Université de Neuchâtel, Suisse
1996-1998	Diplom-Arbeit Direktor: Prof. Dr. Bruno Betschart Titel: Activité anthelmintique de plantes médicinales présentes en Côte d'Ivoire Publikation: « *Prospect for anthelminthic plants in Ivory coast using ethnobotanical criteria* », M.S. Diehl, K. Kamanzi Atindehou, H. Téré and B. Betschart, Journal of ethnopharmacology, 2004, Vol 95, Issues 2-3, p 277-284
1998	Erhalt des Biologie-Diplom, mit „gut"

1998-2002	Studium der Veterinärmedizin an der Universität Zürich, Schweiz NB. Das Biologie-Diplom wurde als Aequivalenz des 1. Jahres bei Bundesamt für Veterinärwesen anerkannt
2002	Eidgenössische Schlussprüfung für Tierärzte an der Universität Zürich, Schweiz

2003-2006	Doktorandin am Institut für Veterinärbakteriologie Zürich der Vetsuisse-Fakultät Universität Zürich, Schweiz Direktor: Prof. Dr. med. vet. Max M. Wittenbrink Titel: Analysis of the PLD gene of *Corynebacterium pseudotuberculosis*