Institut für Veterinär-Bakteriologie der Vetsuisse-Fakultät Universität Bern

(Direktor: Prof. Dr. Joachim Frey)

Arbeit unter der Leitung von

Dr. Carlos Abril

Evaluation of a multiplex real-time PCR for detection of *Listeria monocytogenes* and *Listeria innocua* in clinical brain tissue samples

Inaugural-Dissertation

zur Erlangung der Doktorwürde

der Vetsuisse-Fakultät Universität Bern

vorgelegt von

Andres Fauser

von Steckborn, TG

2009

Von der Vetsuisse-Fakultät Universität Bern auf Antrag
von Prof. Dr. J. Frey als Dissertation genehmigt.

Bern,

Der Dekan der
Vetsuisse-Fakultät Universität Bern

Table of contents

1. Introduction

Bacteria of the genus *Listeria* are small, Gram-positive, rod-shaped organisms that are widely distributed in the environment (Weis and Seeliger, 1975; Farber and Peterkin, 1991; Nufer et al., 2007). The genus *Listeria* consists of six recognized species, of which only *L. monocytogenes* and *L. ivanovii* are considered to be pathogenic. Other *Listeria* species, such as *L. seeligeri* and *L. innocua*, have occasionally been implicated in human and animal disease, respectively (Rocourt et al., 1986; Perrin et al., 2003).

L. monocytogenes, the aethiological agent of listeriosis, is a food-borne pathogen (Farber and Peterkin, 1991). In humans it causes gastroenteritis, encephalitis, abortion and neonatal septicaemia (McLauchlin et al., 2004). Populations at risk are immune-compromised and elderly people, but also pregnant women and newborns. Listeriosis was responsible for the highest hospitalisation and mortality rates amongst known food-borne pathogens in 2007 in European countries including Switzerland (EFSA Journal, 2007).

In ruminants, *L. monocytogenes* causes encephalitis, endometritis and abortion. Septicaemia is frequently observed in newborn animals (McLaughlin et al., 1993) whereas in adult animals listeric septicaemia is relatively rare (Seehusen et al., 2008).

L. ivanovii infections are largely restricted to animals and cause abortions, neonatal sepsis and enteritis in ruminants, in particular sheep (Sergeant et al., 1991; Alexander et al., 1992; Karunasagar et al., 1993; Gerstel et al., 1996; Sahin and Beytut, 2006; Ammendolia et al., 2007). Widespread in the environment and in food, *L. innocua* is considered to be a nonpathogenic bacterium (Perrin et al., 2003). Nonetheless, *L. innocua* has been isolated from brain samples of ruminants with

encephalitis (Walker et al., 1994; Schwaiger et al., 2005) and from a blood sample of a human patient with bacteraemia (Perrin et al., 2003). *L. innocua* from brain samples of ruminants with encephalitis has also been isolated occasionally in our laboratory (unpublished data).

Reliable data on the prevalence of listeriosis are important in order to estimate the role of ruminants as a reservoir and to apply accurate measures for control of listeriosis in animals and prevention of infections in humans. Both depend on the reliability of the methods used for detection and precise identification of the bacteria.

Presumptive diagnosis of encephalitic listeriosis in live animals is generally based on the analysis of clinical signs. Confirmation of listerial infections in the laboratory is currently based on histological examination, immunohistochemistry and bacteriological isolation from brain samples of infected animals (Low and Donachie, 1997; Gasanov et al., 2005). However, these methods are time consuming and false negative results are frequently observed due to low sensitivity (Johnson et al., 1995; OIE Terrestrial Manual 2008).

Several PCR assays have been developed to detect the presence of *Listeria monocytogenes* in food and foodstuff samples (Rodríguez-Lázaro et al., 2004). However, to our knowledge, only one PCR assay has been developed to directly analyze clinical samples from infected animals (Peters et al., 1995).

In this study, we evaluate the use of a multiplex real-time PCR for detection and confirmation of *L. monocytogenes* and *L. innocua* in clinical isolates and in brain tissue samples from animal listerial encephalitis.

2. Materials and methods

2.1. Bacterial strains, growth conditions and lysates

Bacterial strains used in this work are shown in Table 1. Field strains were identified previously by the Swiss National Center for Listeriosis (Centre Hospitalier Universitaire Vaudois CHUV, Lausanne, Switzerland) or by our laboratory using the VITEK® 2 compact system (bioMérieux (Suisse) SA, Geneva, Switzerland). Bacterial strains were cultured on PALCAM agar (Oxoid AG, Pratteln, Basel, Switzerland) at 37°C for 24 hours. Bacterial DNA templates for real-time PCR were obtained by a direct lysis method. Five bacterial colonies were picked and solubilised in 450 ml lysate buffer containing 0.1 M Tris-HCl [pH 8.5], 0.05% Tween 20 and 0.24 mg of proteinase K (Boehringer Mannheim, Mannheim, Germany) per ml and incubated for 60 min at 60 °C followed by inactivation of the proteinase K for 15 min at 97 °C.

2.2. Bacteriological isolation from brain tissue samples

Brain samples from necropsy of 42 bovine, 74 ovine and 40 caprine individuals used in this study were collected between 2001 and 2007 and stored at -20 °C until required for bacterial isolation or DNA extraction. All these brain samples showed typical histopathological lesions of listerial encephalitis and were positive for *Listeria monocytogenes* by immunohistochemistry according to the protocol described by Oevermann et al. (Oevermann et al., 2008). From each brainstem, two subsamples were taken and cultured directly on PALCAM agar and incubated at 37 °C for 24 to 48 hours. Potential *Listeria* sp. colonies were sub-cultivated on Tryptic Soy Agar

(TSA) plates containing 5% sheep blood (Oxoid, Basingstoke, Hampshire, England) to obtain pure cultures and for characterisation of the beta haemolysis.

2.3. DNA extraction from tissue samples and primers and probes

DNA from up to 25 mg of brainstem was extracted by using QIAamp® DNA Mini Kit (QIAGEN, Basel, Switzerland) according to the manufacturer's tissue protocol. DNA was eluted in 100 µl of EL buffer.

Formerly described primers and probes targeting the *listeriolysin O* and *lin02483* genes were used for real-time PCR detection of *L. monocytogenes* and *L. innocua,* respectively (Rodríguez-Lázaro et al., 2004). Specificity of primers and probes was checked using the BLAST database similarity search (www.ncbi.nlm.nih.gov/blast).

Aberrant isolates of *Listeria* do occur and have been described in the literature (Johnson et al., 2004). Moreover, results of the phenotypic analysis with the VITEK2 system of some *Listeria* strains are inconclusive due to low discrimination (Wallet et al., 2005). Therefore, a third highly conserved and specific sequence was chosen for *Listeria* genus identification and used as an endogenous positive control for *Listeria* colony identification (Table 2). The highly conserved sequence region of the aldehyde-alcohol dehydrogenase gene (*aad*) was chosen after comparison of *Listeria* spp. complete genome sequences using the Artemis comparison tool (Wellcome Trust, Sanger Institute, Hinxton, Cambridge, UK) and BLAST database similarity search (www.ncbi.nlm.nih.gov/blast). Primers and a fluorogenic probe for the *aad* gene were designed using the Primer Express software, version 2.0 (Applied Biosystems, Foster City, California).

2.4. Evaluation and application of fluorogenic multiplex real-time PCR for detection of *L. monocytogenes* and *L. innocua* in DNA extracted from brainstem tissue samples

Multiplex real-time amplifications were performed in a total volume of 25 µl, in which 2.5 µl of bacterial lysate or DNA, extracted from brainstem tissue samples, were added to the reaction as template. The real-time PCR reaction contained 12.5 µl of 2X Universal PCR Master Mix, No AmpErase1UNG (Applied Biosystems, Foster City, California), forward and reverse primers at a concentration of 900 nM each and 200 nM probes for detection of both *L. monocytogenes* and *L. innocua*. Additionally, primers and probe for detection of the *aad* gene were added to the reaction to confirm the *Listeria* genus in culture isolates. The primers and probe for the *aad* gene were used at concentrations of 300 nM and 200 nM respectively.

To test DNA extracted from brain tissue samples, primers and fluorogenic probe for the detection of the *aad* gene were not included in the multiplex real-time PCR reaction. For these reactions, primers and probe for the *aad* gene were replaced by an exogenous Internal Positive Control (IPC, Applied Biosystems, Foster City, CA), which was added to each reaction according to the manufacturers' protocol, to check for the presence of eventual PCR inhibitors.

Real-time PCR was performed on a TaqMan® 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) according to the standard thermal cycler protocol. Data were analyzed with the 7500 System SDS Software version 1.2.3f2, and auto settings used to set the baseline. The threshold settings were adjusted to 0.06 for *hly* and 0.02 for *aad*. Standard curves were created according to User bulletin #2: Relative quantitation of gene expression (Applied Biosystems, Foster City, CA).

2.5. Analytical sensitivity and specificity of the multiplex real-time PCR assay

To determine the analytical sensitivity of the multiplex real-time PCR, target sequences of positive control strains were amplified and cloned into pGEM®- T-Easy Vector (Promega Corporation, Madison, WI, USA). The plasmids were purified with the QIAprep® Spin Miniprep Kit (QIAGEN, Basel, Switzerland), and DNA content was measured by spectroscopic analysis and diluted in a 10-fold series to create standard curves (User's Manual, ABI PRISM 7700 Sequence Detection System, Perkin-Elmer Applied Biosystems).

The specificity of the multiplex real-time PCR was tested with 18 collection strains of *L. monocytogenes* isolated from human listeriosis, 12 strains of *L. monocytogenes* from foodstuff or environmental material, 42 *Listeria* species other than *L. monocytogenes* and 20 strains of other bacterial species that are currently found in feed, food or animal infections (Table 1). Additionally, DNA extracted from 11 brain samples of ruminants that presented histological lesions reminiscent of bacterial encephalitis caused by bacteria other than *Listeria* sp. were tested (Table 1).

3. Results

3.1. Bacteriological isolation and identification

A total of 156 brain necropsy samples from 42 bovine, 74 ovine, and 40 caprine individuals were tested by bacterial isolation. Of the 100 isolates obtained, 96 were identified as *L. monocytogenes* using the VITEK® 2 compact system. Four isolates showed low discrimination between *L. monocytogenes* and *L. innocua*. Three of these isolates were phosphatidylinositol-specific phospholipase C (PI-PLC) negative, and one isolate was L-aspartate-arylamidase (AspA) positive. All isolates showed Beta- haemolysis on 5% sheep blood agar, with a single exception. This non-Beta-haemolytic strain was isolated from a sheep brain sample and identified as *L. innocua*.

3.2. Analytical sensitivity and specificity of the *Listeria* multiplex real-time PCR

The detection limits of the *Listeria* multiplex real-time PCR were determined by using a 10-fold dilution series of plasmid preparations containing the *hly*, *lin02483* and *aad* gene target sequences. In two independent experiments, four replicates of each plasmid dilution were tested. The plasmid dilutions covered the range from 10^9 to 1 plasmid molecules per reaction mix. The *Listeria* multiplex real-time PCR was always able to detect 10 copies of each plasmid, and linearity was observed over the range from 10^8 to 10 copies of plasmids. The coefficients of determination for the linear regression were as follows: 0.9947 for *hly*, 0.998 for *lin02483* and 0.9945 for *aad*

As for the specificity of the real-time PCR as determined by analyzing strains of *Listeria* sp. and other bacterial species (Table 1), the *hly* and *lin02483* assays were

100% specific for *L. monocytogenes* and *L. innocua,* respectively. These results are in agreement with a previously published study using the same primer pairs and probes (Rodríguez-Lázaro et al., 2004). For the *aad* assay, all strains of *L. monocytogenes*, *L. welshimeri*, *L. innocua* and *L. seeligeri* were appropriately identified as *Listeria* sp. Additionally, all *L. ivanovii* strains were positively detected, with one exception, the strain CCUG 36672T. *L. grayi* strains were not detected with the *aad* real-time PCR assay (Table 1).

3.3. Evaluation of *Listeria* multiplex real-time PCR sensitivity with brainstem tissue samples and comparison with the sensitivity of bacterial isolation

In total, 156 brainstem tissue samples from ruminants were simultaneously analyzed by the *Listeria* multiplex real-time PCR and by bacterial isolation. Results are summarised in Figure 1. Brainstem samples of 136 animals showed positive results for *L. monocytogenes* (87.18%), and in 20 samples no signal was detected either for *L. monocytogenes* or for *L. innocua* (12.82%) by *Listeria* multiplex real-time PCR.
L. monocytogenes was isolated from 100 of the 156 analyzed brainstem samples (64.10%), and *L. innocua* was isolated from a bovine brainstem only once, while 55 brainstem samples remained negative by bacterial isolation (35.26%).

Samples from 98 animals were found to be positive and 17 samples negative by both bacterial isolation and real-time PCR (Figure 1b). Three brainstem samples were positive by bacterial isolation but negative by real-time PCR. The isolates of these three brainstem samples were subsequently tested by real-time PCR. Two were positive for *L. monocytogenes* and one for *L. innocua* by real-time PCR. Furthermore, real-time PCR was able to additionally detect 38 positive animals that were negative by bacterial isolation (Figure 1b).

4. Discussion

In this study, we evaluated the use of a multiplex real-time PCR assay for the detection of *L. monocytogenes* and *L. innocua* in brain samples of infected ruminants. Two specific fluorogenic probes, one FAM-labeled and one Cy5-labeled, were used for the detection of *L. monocytogenes* and *L. innocua,* respectively. The *aad* Cy3-labeled fluorogenic probe allowed identification of the genus *Listeria* in culture isolates and was additionally used as an internal positive control. For testing of brain tissue samples, a commercial exogenous internal positive control was preferred in order to exclude internal inhibition. Loss of signal was restricted to the commercial internal positive control or to the *aad*-probe in cases where the sample tested was positive for *L. monocytogenes* or *L. innocua*. The reporter and quencher dyes used in this study allowed the simultaneous detection of three targets in the same reaction without overlapping of fluorescence signal. ROX^{TM} dye, which is included in the TaqMan® Universal PCR Master Mix, was used as a passive reference in the reaction to normalise fluorescence signals of the reporter dyes.

The *Listeri*a real-time PCR was found to be highly sensitive and specific (100%) when tested using plasmids containing the target genes and using *L. monocytogenes* strains and *Listeria* non-*monocytogenes* strains. The PCR targeting the highly conserved region of the *aad* gene (identified *Listeria* species using Artemis comparison tool (Wellcome Trust, Sanger Institute, Hinxton, Cambridge, UK) and BLAST database similarity search (www.ncbi.nlm.nih.gov/blast), could not detect any *L. grayi*, strain used in this study. This lack of amplification could occur due to a mutation at the primer sites, resulting in PCR negative results.

For performance evaluation of the *Listeria* multiplex real-time PCR, brainstem tissue samples from ruminants with listerial encephalitis and encephalitis caused by

other aetiological agents were used in this study. In order to compare results of bacterial isolations with results of real-time PCR, this study employed brain tissue samples showing typical histological lesions of listerial encephalitis that were also positive for *Listeria* by immunohistochemistry. Interestingly, real-time PCR confirmed the presence of *L. monocytogenes* in 87% of the samples from cases of encephalitic listeriosis, but in comparison, bacterial isolation detected only 64%. Additionally, no cross-reactivity was observed when brain tissue samples with histopathological lesions caused by other aetiological agents were tested by real-time PCR, showing a high specificity of the direct real-time PCR method.

The 13% of samples that originated from brains positively identified for listeriosis by immunohistochemistry and were found to be negative by real-time PCR might originate from samples that did not include enough of the observed micro-abscesses or maybe none at all, thus lacking sufficient *Listeria* material to be detected by real-time PCR.

The uneven distribution of cerebral microabscesses in certain animals must be taken into consideration when examining necropsy samples by real-time PCR or bacterial culture. This is certainly demonstrated by the following situation: three animals, which were positive by bacterial isolation, were negative by the real-time PCR, even after three consecutive DNA extractions (Figure 1). This observation is in concordance with reports of other authors (Loeb, 2004) and affects only a minor subset of the samples analyzed. However, it also must be noted that in the study of Loeb, only 80.9% of the brains with typical histopathological lesions for listeriosis could be confirmed by immunohistochemistry. The remaining cases were either false negative results or may have included all possible differential diagnoses.

The 24% of samples that were positively identified by real-time PCR but were negative by bacterial culture may be explained by pretreatment with antibiotics or by the long storage time at -20°C.

In summary, this study describes a method of fast, highly sensitive and specific real-time PCR for diagnosis of *Listeria monocytogenes* in brainstem tissue samples of infected ruminants.

5. References

Alexander, A.V., Walker, R.L., Johnson, B.J., Charlton, B.R., Woods, L.W., 1992. Bovine abortions attributable to *Listeria ivanovii*: four cases (1988-1990). J. Am. Vet. Med. Assoc. 200, 711-714.

Ammendolia, M.G., Superti, F., Bertuccini, L., Chiarini, F., Conte, M.P., Cipriani, D., Seganti, L., Longhi, C., 2007. Invasive pathway of *Listeria ivanovii* in human amnion-derived WISH cells. Int. J. Immunopathol. Pharmacol. 20, 509-518.

Farber, J.M., Peterkin, P.I., 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. 55, 476-511.

Gasanov, U., Hughes, D., Hansbro, P.M., 2005. Methods for the isolation and identification of *Listeria* spp. and *Listeria monocytogenes*: a review. FEMS Microbiol. Rev. 29, 851-875.

Gerstel, B., Gröbe, L., Pistor, S., Chakraborty, T., Wehland, J., 1996. The ActA polypeptides of *Listeria ivanovii* and *Listeria monocytogenes* harbor related binding sites for host microfilament proteins. Infect. Immun. 64, 1929-1936.

Johnson, G.C., Fales, W.H., Maddox, C.W., Ramos-Vara, J.A., 1995. Evaluation of laboratory tests for confirming the diagnosis of encephalitic listeriosis in ruminants. J. Vet. Diagn. Invest. 7, 223-228.

Johnson, J., Jinneman, K., Stelma, G., Smith, B.G., Lye, D., Messer, J., Ulaszek, J., Evsen, L., Gendel, S., Bennett, R.W., Swaminathan, B., Pruckler, J., Steigerwalt, A., Kathariou, S., Yildirim, S., Volokhov, D., Rasooly, A., Chizhikov, V., Wiedmann, M., Fortes, E., Duvall, R.E., Hitchins, A.D., 2004.

Natural atypical *Listeria innocua* strains with L*isteria monocytogenes* pathogenicity island 1 genes. Appl. Environ. Microbiol. 70, 4256-4266.

Karunasagar, I., Krohne, G., Goebel, W., 1993. *Listeria ivanovii* is capable of cell-to-cell spread involving actin polymerization. Infect. Immun. 61, 162-169.

Loeb, E., 2004. Encephalitic listeriosis in ruminants: immunohistochemistry as a diagnostic tool. J. Vet. Med. A Physiol. Pathol. Clin. Med. 51, 453-455.

Low, J.C., Donachie, W., 1997. A review of *Listeria monocytogenes* and listeriosis. Vet. J. 153, 9-29.

McLauchlin, J., Mitchell, R.T., Smerdon, W.J., Jewell, K., 2004. *Listeria monocytogenes* and listeriosis: a review of hazard characterisation for use in microbiological risk assessment of foods. Int. J. Food Microbiol. 92, 15-33.

McLaughlin, B.G., Greer, S.C., Singh, S., 1993. Listerial abortion in a llama. J. Vet. Diagn. Invest. 5, 105-106.

Nufer, U., Stephan, R., Tasara, T., 2007. Growth characteristics of *Listeria monocytogenes*, *Listeria welshimeri* and *Listeria innocua* strains in broth cultures and a sliced bologna-type product at 4 and 7 ºC. Food Microbiol. 24, 444-451.

Oevermann, A., Botteron, C., Seuberlich, T., Nicolier, A., Friess, M., Doherr, M.G., Heim, D., Hilbe, M., Zimmer, K., Zurbriggen, A., Vandevelde, M., 2008. Neuropathological survey of fallen stock: active surveillance reveals high prevalence of encephalitic listeriosis in small ruminants. Vet. Microbiol. 130, 320-329.

Perrin, M., Bemer, M., Delamare, C., 2003. Fatal case of *Listeria innocua* bacteremia. J. Clin. Microbiol. 41, 5308-5309.

Peters, M., Pohlenz, J., Jaton, K., Ninet, B., Bille, J., 1995. Studies of the detection of Listeria monocytogenes by culture and PCR in cerebrospinal fluid samples from ruminants with listeric encephalitis. Zentralbl. Veterinarmed. 42, 84-88.

Rocourt, J., Hof, H., Schrettenbrunner, A., Malinverni, R., Bille, J., 1986. [Acute purulent *Listeria seelingeri* meningitis in an immunocompetent adult]. Schweiz. Med Wochenschr. 116, 248-251.

Rodríguez-Lázaro, D., Hernández, M., Scortti, M., Esteve, T., Vázquez-Boland, J.A., Pla, M., 2004. Quantitative detection of *Listeria monocytogenes* and *Listeria innocua* by real-time PCR: assessment of *hly*, *iap*, and *lin*02483 targets and AmpliFluor technology. Appl. Environ. Microbiol. 70, 1366-1377.

Sahin, M., Beytut, E., 2006. Abortions in sheep due to *Listeria ivanovii* in the Kars region. Turk. J. Vet. Anim. Sci. 30, 503-506.

Schwaiger, K., Stierstorfer, B., Schmahl, W., Lehmann, S., Gallien, P., Bauer, J., 2005. [The incidence of bacterial CNS infections in roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*) and chamois (*Rupicapra rupicapra*) in Bavaria]. Berl. Munch. Tierarztl. Wochenschr. 118, 45-51.

Seehusen, F., Lehmbecker, A., Puff, C., Kleinschmidt, S., Klein, S., Baumgärtner, W., 2008. *Listeria monocytogenes* septicaemia and concurrent clostridial infection in an adult alpaca (*Lama pacos*). J. Comp. Pathol. 139, 126-129.

Sergeant, E.S., Love, S.C., Mcinnes, A., 1991. Abortions in sheep due to *Listeria ivanovii*. Aust. Vet. J. 68, 39.

Walker, J.K., Morgan, J.H., McLauchlin, J., Grant, K.A., Shallcross, J.A., 1994. *Listeria innocua* isolated from a case of ovine meningoencephalitis. Vet. Microbiol. 42, 245-253.

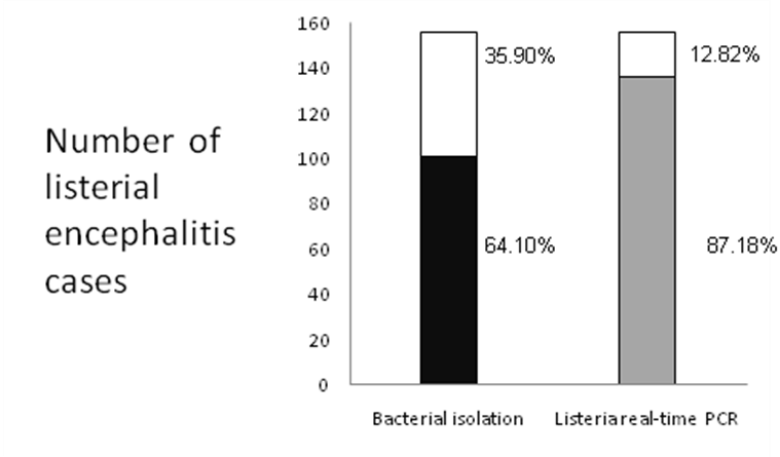
Wallet, F., Loiez, C., Renaux, E., Lemaitre, N., Courcol, R.J., 2005. Performances of VITEK 2 colorimetric cards for identification of gram-positive and gram-negative bacteria. J. Clin. Microbiol. 43, 4402-4406.

Weis, J., Seeliger, H.P., 1975. Incidence of *Listeria monocytogenes* in nature. Appl. Microbiol. 30, 29-32.

6. Figures

Figure 1. Brain tissue samples from 156 ruminants with listerial histopathological lesions and positive by immunohistochemistry were analyzed by bacterial isolation and the *Listeria* real-time PCR. (A) Total Number of listerial encephalitis cases confirmed by bacterial isolation: ■, and by the *Listeria* multiplex real-time PCR: ■. (B) Cross tabulation of results obtained by bacterial isolation and the *Listeria* multiplex real-time PCR.

A



B

Multiplex real-time PCR	Isolation +	Isolation -
+	**98**	**38**
-	**3**	**17**

7. Tables

Table 1. *Listeria* and non-*Listeria* strains used in this study

Bacteria species	Strain no.[a]	Clinical origin	Country of origin	multiplex real time PCR result for[b]:		
				hly	*lin02483*	*aad*
Listeria strains						
L. monocytogenes	CHUV 016/2007	LCR	Switzerland	+	-	+
	CHUV 162/2006	LCR	Switzerland	+	-	+
	CHUV 083/2006	LCR	Switzerland	+	-	+
	CHUV 072/2006	LCR	Switzerland	+	-	+
	CHUV 052/2006	LCR	Switzerland	+	-	+
	CHUV 037/2006	LCR	Switzerland	+	-	+
	CHUV 227/2005	LCR	Switzerland	+	-	+
	CHUV 212/2005	LCR	Switzerland	+	-	+
	CHUV 200/2005	LCR	Switzerland	+	-	+
	CHUV 009/2007	Neonatal infection	Switzerland	+	-	+
	CHUV 176/2006	Neonatal infection	Switzerland	+	-	+
	CHUV 141/2006	Neonatal infection	Switzerland	+	-	+
	CHUV 128/2006	Neonatal infection	Switzerland	+	-	+
	CHUV 253/2005	Neonatal infection	Switzerland	+	-	+
	CHUV 203/2005	Neonatal infection	Switzerland	+	-	+
	CHUV 092/2005	Neonatal infection	Switzerland	+	-	+
	CHUV 091/2005	Neonatal infection	Switzerland	+	-	+
	CHUV 053/2005	Neonatal infection	Switzerland	+	-	+
	CHUV 055/2007	Food	Switzerland	+	-	+
	CHUV 022/2007	Food	Switzerland	+	-	+
	CHUV 019/2007	Food	Switzerland	+	-	+
	CHUV 007/2007	Food	Switzerland	+	-	+
	CHUV 005/2007	Food	Switzerland	+	-	+
	CHUV 181/2006	Food	Switzerland	+	-	+
	CHUV 003/2005	Environment	Switzerland	+	-	+
	CHUV 014/2005	Environment	Switzerland	+	-	+
	CHUV 026/2005	Environment	Switzerland	+	-	+
	CHUV 031/2005	Environment	Switzerland	+	-	+
	CHUV 153/2005	Environment	Switzerland	+	-	+
	CHUV 188/2005	Environment	Switzerland	+	-	+
L. ivanovii	CCUG 15528T	Sheep	Bulgaria	-	-	+
	CCUG 24940	Human	France	-	-	+
	CCUG 36672T	Food	France	-	-	-
	ATCC 19119	Sheep	Bulgaria	-	-	+
	ZOBA CA-3	Environment	Switzerland	-	-	+
	ZOBA CA-4	Environment	Switzerland	-	-	+
	CHUV 169/2006	Unknown	Switzerland	-	-	+
	CHUV 48/2007	Unknown	Switzerland	-	-	+
	CHUV 145/2005	Unknown	Switzerland	-	-	+
	CHUV 43/2007	Unknown	Switzerland	-	-	+
	CHUV 244/2005	Unknown	Switzerland	-	-	+
	CHUV 125/2004	Unknown	Switzerland	-	-	+
L. welshimeri	CCUG 15529T	Decaying vegetation	USA	-	-	+
	CCUG 24934	Environment	USA	-	-	+
	CCUG 24935	Dairy Products	France	-	-	+
	CHUV 47/2004	Unknown	Switzerland	-	-	+
	CHUV 71/2004	Unknown	Switzerland	-	-	+
	CHUV 170/2006	Unknown	Switzerland	-	-	+
	CHUV 56/2005	Unknown	Switzerland	-	-	+
	CHUV 44/2004	Unknown	Switzerland	-	-	+

Continued on next page

Table 1–*Continued*

Bacteria species	Strain no.[a]	Clinical origin	Country of origin	multiplex real time PCR result for[b]:		
				hly	*lin02483*	*aad*
Listeria strains (continuation)						
L. innocua	CCUG 15531T	Cattle brain	France	-	+	+
	CCUG 21830	Cheese	Sweden	-	+	+
	CCUG 24924	Food	France	-	+	+
	CHUV 48/2004	Unknown	Switzerland	-	+	+
	CHUV 229/2005	Unknown	Switzerland	-	-	+
	CHUV 20/2005	Unknown	Switzerland	-	+	+
	CHUV 50/2004	Unknown	Switzerland	-	+	+
	CHUV 45/2004	Unknown	Switzerland	-	+	+
	CHUV 178/2005	Unknown	Switzerland	-	+	+
	CHUV 175/2005	Unknown	Switzerland	-	+	+
	CHUV 219/2005	Unknown	Switzerland	-	+	+
	CHUV 67/2004	Unknown	Switzerland	-	+	+
	CHUV 196/2005	Unknown	Switzerland	-	+	+
	CHUV 214/2005	Unknown	Switzerland	-	+	+
	CHUV 71/2005	Unknown	Switzerland	-	-	+
L. seeligeri	CCUG 15530T	Soil	Germany	-	-	+
	CCUG 24929	Food	France	-	-	+
	CCUG 27609	Cheese	Sweden	-	-	+
	CHUV 35/2008	Unknown	Switzerland	-	-	+
L. grayi	CCUG 24933	Human faeces	Italy	-	-	-
	CCUG 4983T	Feces of Chinchilla	Denmark	-	-	-
	CCUG 4984	Corn stalks and leaves	USA	-	-	-
Non-*Listeria* strains						
Bacillus subtilis	DSM 618	Unknown	Unknown	-	-	-
Citrobacter freundii	NZ 3872/90	Human faeces	Switzerland	-	-	-
Enterobacter cloacae	K/M 149/05	Dog joint	Switzerland	-	-	-
Klebsiella pneumoniae	O/D 1128/04	Swine liver	Switzerland	-	-	-
Pseudomonas aeruginosa	ATTC 27853	Human bloodculture	Unknown	-	-	-
Enterococcus faecalis	DSMZ 20478	Unknown	Unknown	-	-	-
Enterococcus faecalis	Mon 973/07	Poultry cloaca	Switzerland	-	-	-
Enterococcus faecium	DSM 20477	Unknown	Unknown	-	-	-
Enterococcus hirae	DSM 20160	Unknown	Unknown	-	-	-
Lactobacillus oris	IMD 718/07	Parrot	Switzerland	-	-	-
Lactobacillus garviae	DSM 20684	Cattle mastitis	Unknown	-	-	-
Lactobacillus lactis	DSM 20481	Unknown	Unknown	-	-	-
Rhodococcus equi	D 205/95	Horse	Switzerland	-	-	-
Staphylococcus aureus	ATCC 25923	Unknown	Unknown	-	-	-
Staphylococcus epidermidis	ATCC 14990	Human nose	Unknown	-	-	-
Streptococcus agalactiae	DSM 2134	Unknown	Unknown	-	-	-
Streptococcus oralis	K/M 375/06	Cat pharynx	Switzerland	-	-	-
Streptococcus uberis	M 426/04	Cattle milk	Switzerland	-	-	-
Arcanobacter pyogenes	K/M 1385/06	Swine abdominal cavity	Switzerland	-	-	-
Erysipelothrix rhusiopathiae	K/M 1846/06	Sheep synovia	Switzerland	-	-	-

[a]ATCC, American Type Culture Collection, Manassas, VA, USA; CCUG, Culture Collection of the University of Göteborg, Sweden; CHUV, Centre Hospitalier Universitaire Vaudoise, Lausanne, Switzerland; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GMbH, Braunschweig, Germany; K/M, M and O/D, Institute of Veterinary Bacteriology, ZOBA, Vetsuisse Faculty, Bern, Switzerland.

[b]Qualitative results of the multiplex real-time PCR *L. monocytogenes* (*hly*), *L. innocua* (*lin02483*) and *Listeria* spp. (*aad*): +, positive; -, negative.

Table 2. Primers and probes used for fluorogenic (TaqMan®) PCR

Target gene	Name of primers and probes	Nucleic acid sequences of primers and probes (5'-3') with probe dyes	Reference
hly	hlyQF	CATGGCACCACCAGCATCT	Rodríguez-Lázaro et al., 2004
	hlyQR	ATCCGCGTGTTTCTTTTCGA	Rodríguez-Lázaro et al., 2004
	hlyQP	**FAM**-CGCCTGCAAGTCCTAAGACGCCA-**TAMRA**	Rodríguez-Lázaro et al., 2004
lin02483	lipHQF	ACCCGGGCCGCTTATGA	Rodríguez-Lázaro et al., 2004
	lipHQR	CGAACGCAATTGGTCACG	Rodríguez-Lázaro et al., 2004
	lipHQP	**Cy-5**-TTCGAATTGCTAGCGGCACACCAGT-**BHQ-2**	Rodríguez-Lázaro et al., 2004
aad	LapTM-F	CAAAATGTAAGTGCGACTAACTTGCT	In this study
	LapTM-R	TGGAAGTTTGAACCATTGCATATT	In this study
	LapTM-P	**Cy-3**-TTTCTCCGATCCGCGATACGTTTAACGT-**BHQ-2**	In this study

8. Acknowledgments

I would like to thank all the people who made a contribution to this project, especially:

Dr. Carlos Abril and Dr. Anna Oevermann, for giving me the opportunity to work on this project and for their support and valuable advice during the whole project,

Prof. Dr. Joachim Frey, for his support and critical thoughts,

Isabelle Brodard, for her excellent technical assistance,

Prof. Dr. Jacques Bille, for providing us with *Listeria* strains,

Dr. Vladimira Hinic, for introducing me to the lab,

Dr. Edy Vilei, for his help in editing the manuscript,

Dr. Daniela Hüssy, for her advice and encouragement,

all the members of the Institute for Veterinary Bacteriology,

and my partner and family, for their understanding and support.