4. Results

4.1 Macroscopic and microscopic examinations

4.1.1 Lymph nodes without typical pathoanatomical lesion.

Total of 703 lymph nodes were collected from clinically healthy pigs from slaughterhouses in Nohra (390 lymph nodes from 78 pigs), Meiningen (215 lymph nodes from 43 pigs) and Freiburg (98 lymph nodes from 8 pigs) and were macroscopically examined. None of them showed any typical pathoanatomic lesion of mycobacteriosis (granulomatous and caseous mass) but the evidences of non-specific pathoanatomical lesions such as various degrees of hyperemia to haemorrhage and abscess were noted in 42 and 2 lymph nodes respectively.

All lymph node materials was stained with Ziehl-Neelsen stain and examined under light microscope at 1000 times magnification as recommended in table 1. Acid-fast bacilli were visualized in 35 from 703 samples (4.97 %) and nearly all of them contain acid fast bacilli in low to moderate degree (from 1+ to 2+).

4.1.2 Lymph nodes with pathoanatomical lesions.

139 lymph nodes were collected from clinically healthy pigs from slaughterhouses in Essen/Clopp. (125 lymph nodes from 88 pigs) and Freiburg (14 lymph nodes from 8 pigs) and examined macroscopically. Pathoanatomic lesions for mycobacteriosis found varied from pin-point to bigger than 5 mm in size and from single to multifocal lesions. None of them showed any signs of calcification. The predominant change observed was the multifocal

caseous masses in size of 2 to 3 mm. Various degrees of hyperemia to haemorrhages were found in 9 lymph nodes.

By means of Ziehl-Neelsen stain, a stained smear from each lymph node was observed under light microscope at 1000 times magnification, AFB were found in 94 direct smear slides from lymph node materials. The moderate to high degree (2+ to 4+) of acid fast bacilli predominated among the population of AFB found lymph nodes (78 from 94) as shown in table 3.

Table 3. The number of AFB observed in lymph node materials.

	0	**±**	**1+**	**2+**	**3+**	**4+**
LN. w/o. lesion	657	11	12	22	1	0
LN. w. lesion	15	26	16	31	30	17

LN = Lymph node; w/o = without; w. = with; lesion = typical pathoanatomic lesion for mycobacteriosis

4.2 Bacterial culture

The first colonies were observed after 2 weeks in some tubes but most cultures showed the first colonies within 5-6 weeks. Table 4 shows the numbers of tubes and time periods, in which first colony was observed throughout the incubation period.

Table 4. Incubation time needed for first colonies observed on culture media.

	1st–2nd Wk	3rd-4th Wk	5th-6th Wk	7th-8th Wk	9th-10th Wk	11th-12th Wk
Ogawa	3 (1)	18 (4)	71	38	14	0
LJ	7 (6)	12 (4)	26	40	14	0
Stonebrink	0	10 (2)	47	19	4	0

Ogawa = Ogawa culture media with glycine and PACT; LJ = Löwenstein-Jensen culture media; Stonebrink = Stonebrink culture media with PACT; (x) = number of non mycobacterium isolate confirmed by PCR

After 12 weeks of observation, 78 isolates of bacteria were harvested from 139 lymph nodes with lesions (56.11 %). These were related to 69.79% of the animals (67 from 96 pigs). From lymph nodes without any specific lesion, 97 bacterial isolates were harvested from the total of 703 lymph nodes (13.79%) and related to 42.14% of the animal (51 from 121 pigs).

4.3 PCR

DNA from each of 177 isolates was extracted from a single colony from each tube. Every DNA sample was tested by “duplex PCR”, which contained primers for IS 901 and IS 1245 to determine *M. avium* ssp. *avium* and *M. avium* ssp. *hominissuis.* Figure 1 shows the eletrophoretic picture obtained from Duplex PCR with IS 901 and IS 1245 specific primer systems. Lane 1 and 2 show 426 bp DNA band of IS 1245, which is specific for *M. avium* ssp. *hominissuis*, whereas 426 and 1108 bp DNA bands were obtained from *M.avium* ssp. *avium* as showed in lane 5 and 6. Lane 7, 8 and 9 are distilled water as negative control*, Mycobacterium avium* ssp. *avium* positive control DSM 44156 and molecular weight marker, respectively.

The negative samples were tested by 2 single PCR systems to detect FR 300 and 16S rDNA for *M. intracelluare* and *Mycobacterium* spp*.* respectively.

Out of 175 bacterial isolates from pig lymph nodes 147 strains (84.0%) belong to MAIC, 11 were non MAIC strains (6.28%) and reacted with genus specific primers but negative with IS 1245, IS 901 and FR 300 primers. Seventeen strains (9.71%), which did not react with any mycobacteria-specific primers were classified as non-*mycobacterium* strains. Table 5 shows PCR results of bacterial strains isolated from pig lymph nodes.

From 152 wildlife animal lymph odes collected during 2002-2003, 2 (14.47%) were cultured and confirmed by PCR as MAIC member (15 *M. a. avium*, 6 *M. intracellulare* and 1 *M. a. hominissuis*).

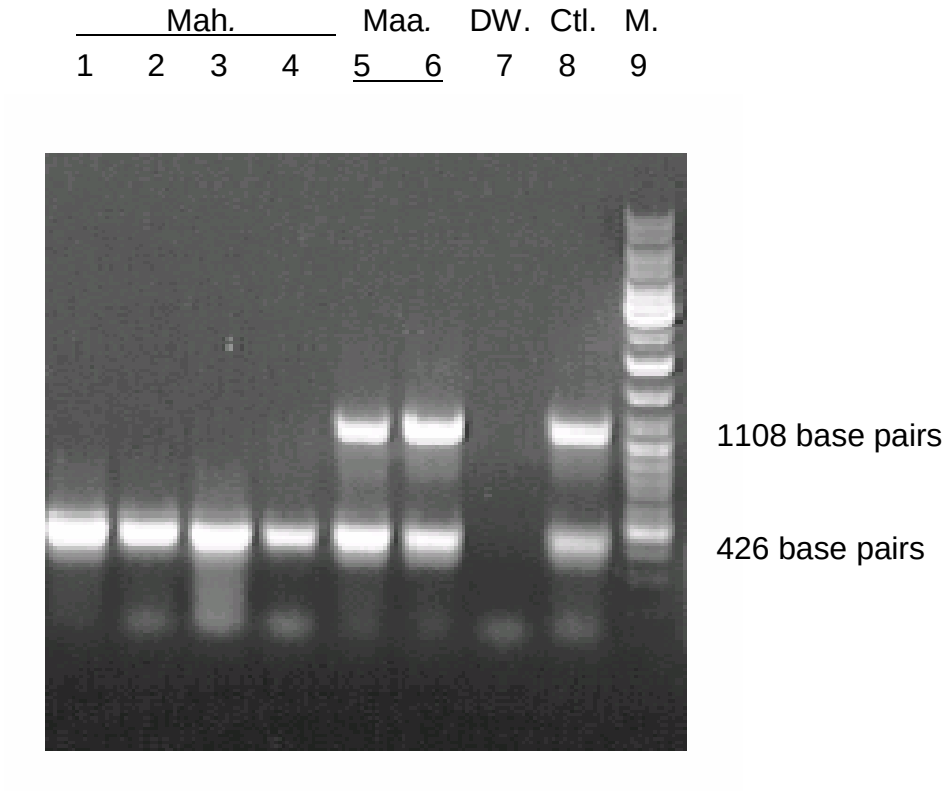

Fig. 1. PCR products generated from MAIC strains DNA by Duplex PCR targeting IS1245 and IS901

Maa.= *Mycobacterium avium* ssp.*avium*; Mah.= *Mycobacterium avium* ssp. *hominissuis*; DW.= Distilled water; Ctl.= DSM 44156 as positive control; M.= Molecular weight marker

Table 5. MAIC isolates from pig lymph nodes confirmed by PCR.

	Number of animals	Culture pos. (%)	Number of LN	Culture pos. (%)	Maa (%)	Mah (%)	Mi (%)	Other Mb (%)	Other genus (%)
Meiningen									
w. path. lesion	0	0	0	0	0	0	0	0	0
w/o path lesion	43	26	215	52	0	42	1	7	2
	43	**60.46%**	**215**	**24.18%**	0%	80.77%	1.92%	13.46%	3.85%
Nohra									
w. path. lesion	0	0	0	0	0	0	0	0	0
w/o path lesion	78	25	390	41	5	16	1	6	13
	78	**32.05%**	**390**	**10.51%**	12.19%	39.02%	2.43%	14.63%	31.70%
Essen									
w. path. lesion	88	61	125	74	8	64	0	0	2
w/o path lesion	0	0	0	0	0	0	0	0	0
	88	**69.31%**	**125**	**59.20%**	10.81%	86.49%	0%	0%	2.70%
Freiburg									
w. path. lesion	8	6	14	6	0	5	0	1	0
w/o path lesion	0	0	98	2	0	2	0	0	0
	8	**75%**	**112**	**7.20%**	0%	87.5%	0%	12.50%	0%

Maa *= Mycobacterium avium* ssp. *avium*; Mah = *Mycobacteriun avium* ssp. *hominissuis*; Mi *= Mycobacterium intracellulare*; Mb = *Mycobacterium spp*; pos. = positive; w. = with; w/o = without; LN = Lymph nodes; path.lesion = pathoanatomical lesion

The following tables (6a,6b) show the number of MAIC sorted by geographic origins of pigs.

Table 6a. Number of pigs with mycobacteria sorted by geographic origin.

	Maa	Mah	Mi	Other MAIC	Other genus	Mixed culture
BW	0	5	0	0	0	1
NI	6	29	0	0	0	3
NRW	0	11	0	0	0	0
SA	0	1	0	0	0	1
SH	1	0	0	0	0	0
TH	1	27	3	4	7	8
Holl	0	10	0	0	0	0
Total	**8**	**83**	**3**	**4**	**7**	**13**

Maa = *Mycobacterium avium* ssp. *avium;* Mah = *Mycobacterium avium* ssp. *hominissuis;* Mi = *Mycobacterium intracellulare;* BW = Baden-Württemberg; NI = Lower Saxony; = NRW = North Rhine-Westphalia; SA = Saxony-Anhalt; SH = Schleswig-Holstein; TH = Thuringia; Holl = The Netherlands.

Table 6b. Numbers of mycobacterial isolates confirmed by PCR sorted by geographic origin of pigs.

	Maa	Mah	Mi	Other Mb	Other genus
BW	0	7	0	1	0
NI	8	40	0	2	3
NRW	0	13	0	0	0
SA	0	2	0	1	2
SH	1	0	0	0	0
TH	4	55	5	7	12
Holl	0	11	0	0	0
Total	**13**	**129**	**5**	**11**	**17**

Maa = *Mycobacterium avium* ssp. *avium;* Mah = *Mycobacterium avium* ssp. *hominissuis;* Mi = *Mycobacterium intracellulare;* Mb = *Mycobacterium* spp.; BW = Baden-Württemberg; NI = Lower Saxony; = NRW = North Rhine-Westphalia; SA = Saxony-Anhalt; SH = Schleswig-Holstein; TH = Thuringia; Holl = The Netherlands.

The following tables (7, 8, 9 and 10) show the distribution of MAIC isolated from multi-organ MAIC infection grouped by slaughter houses.

Table 7. Distribution of mycobacteria isolated from various lymph nodes of pigs from Nohra.

Animal number.	Mesen.	Tracheo.	Mandi.	Subiliaci.	Poplitei.
1	Mah				
2			Mah		
3		Mah			
6	Mah	Mi		non Mb	
10				non Mb	
12				non Mb	
13		Mah		non Mb	
14	non MAIC	non MAIC			non Mb
25		Maa			
29	Mah				
30				Mah	
57	Mah				
58		non Mb			
62	Maa				
64	Maa				Mah
65	Mah	Mah	Maa	Mah	Mah
66		Mah	Mah	Mi	
67				non Mb	
68	Mi				
69	Mi		Mi	Mi	non MAIC
70		non MAIC			
76			non Mb		
116			Mah		
120				Maa	
121		Mah	Mah		

Mesen.= *Ln. mesenterialis;* Tracheo.= *Lnn. tracheobronchiales ;* Subiliaci. = *Lnn. subiliaci ;* Poplitei*.* = *Lnn. poplitei;* Mandi.=*Ln. mandibularis.* Maa = *Mycobacterium avium* ssp. *avium;* Mah = *Mycobacterium avium* ssp. *hominissuis;* Mi = *Mycobacterium intracellulare;* MAIC = *Mycobacterium avium- intracellulare* complex.

Table 8. Distribution of MAIC isolated from various lymph nodes of pigs from Meiningen.

Animal number	Mesen.	Tracheo.	Subiliaci	Poplitei.	Mandi.
32		non MAIC			
33					non Mb
35	Mah				
36	Mi				
37	Mi				
39			non MAIC	non MAIC	
44			non Mb		Mi
45					non MAIC
47	Mah				
85	Mah				
86	Mah	Mah	Mah		
87		Mah			
88	Mah	Mah			Mah
89		Mah			
90	Mah	Mah	Mah	Mah	Mah
91	Mah			Mah	Mah
92	Mah	Mah			Mah
93	Mah	Mi			
95	Mah	Mah			Mah
96	Mah	Mah			Mah
97	Mah	Mah			
98	Mah	Mah			Mah
99	Mah	Mah			Mah
101		Mah		Mah	Mah
102		Mah			
107		Mah			

Mesen.= *Ln. mesenterialis;* Tracheo.= *Lnn. tracheobronchiales ;* subiliaci. = *Lnn. Subiliaci ;* Poplitei*.* = *Lnn. poplitei;* Mandi.= *Ln. mandibularis.* Maa = *Mycobacterium avium* ssp. *avium;* Mah = *Mycobacterium avium* ssp. *hominissuis;* Mi = *Mycobacterium intracellulare;* MAIC = *Mycobacterium avium- intracellulare* complex.

Animal number	Mesen.	Tracheo.	Mandi.
127			Maa
129			Mah
131			Mah
132			Mah
133			Mah
134			Mah
136			Mah
137			Mah
139			Mah
140			Mah
141			Mah
142			Mah
145			Mah
149			Mah
151			Mah
152			Mah
153			Mah
154			Mah
158			Mah
160			Mah
161			Maa
162		Mah	Mah
163			Mah
164			Mah
165			Mah
166	Mah		
167			Mah
169		Mah	Mah
172	Mah		Mah
173	Mah		Mah
174			Mah
175			Mah
176	Maa		
177			Maa
178		Maa	Maa
179	Maa	Maa	
180		non Mb	Mah
181			Mah
182	Mah	non Mb	Mah
183			Mah
184	Mah		Mah
185			Mah
189	Mah		Mah
190			Mah
191			Mah
193			Mah
194	Mah		Mah
196			Mah
197			Mah
198	Mah		Mah
199			Mah
200			Mah
203			Mah
204			Mah
205			Mah
206			Mah
207			Mah
208			Mah
209		Mah	
210			Mah
213			Mah

Table 9. Distribution of MAIC isolated from various lymph nodes of pigs from Essen/Clopp..

Mesen.=*Ln.mesenterialis;*
Tracheo.=*Lnn.tracheobronchiale*
Mandi.=*Lnn. mandibularis;*
Maa=*Mycobacterium avium* ssp. *avium;*
Mah = *Mycobacterium avium* ssp. *hominissuis;*
Mi =*Mycobacterium intracellulare;* Mb = *Mycobacterium spp.*

Table 10. Distribution of MAIC isolated from various lymph nodes of pigs from Freiburg.

Animal number	Mesen.	Retro.	Tracheo.	Subiliaci	Poplitei.	Mandi.
113						non MAIC
114						Mah
215			Mah			Mah
216						Mah
217						Mah
217		Mah				Mah

Mesen.= *Ln. mesenterialis;* Tracheo.= *Lnn. tracheobronchiales ;* Subiliaci. = *Lnn. subiliaci ;* Poplitei*.* = *Lnn. poplitei;* Mandi.= *Ln. mandibularis.* Maa = *Mycobacterium avium* ssp. *avium;* Mah = *Mycobacterium avium* ssp. *hominissuis;* Mi = *Mycobacterium intracellulare;* MAIC = *Mycobacterium avium- intracellulare* complex.

4.4 PFGE

After chromosomal DNA digestion by restriction enzyme *Xba* I, band patterns from 11 to 22 bands were observed from every MAIC isolate. Sixty three different patterns were demonstrated from 106 isolates of *M. avium* ssp. *avium* and *M. avium* ssp. *hominissuis* as shown in figures 2 and 3.

One characteristic PFGE pattern was generated from 34 pigs from Meiningen ("ecological" in Thuringia, Germany) and Essen/Clopp. (conventional farms Lower Saxony, North Rhine-Westphalia and the Netherlands) and three foxes (Thuringia, Germany), which was detected before only in 2000 from cattle from Cloppenburg, Niedersachsen, Germany according to the lab data of BfaV in Jena, Germany. Only small variations within this group could be observed. This was the first time since 2000 that Cloppenburg-like DNA band

pattern were detected from pig isolates in large scale both in terms of animal numbers and geographic distribution (Moser and Werner, non published).

The dendrogramme of the Cloppenburg-like DNA pattern *M. avium* ssp. *hominissuis* of pigs and cattle are shown in figure 5 and 6.

On the other hand, no clear evidence of the same characteristic DNA band pattern of animals from the same geographic origin was observed.

4.5 RFLP

IS 1245 fingerprints of pig MAIC isolate showed highly diverse RFLP band patterns, which could be divided into two groups. The first group, *M. avium* ssp. *avium* isolates, showed distinct RFLP patterns of 2- and 3-band. The second group demonstrated a highly polymorphic pattern of multiband characteristics for *M. avium* ssp. *hominissuis* (from 5 to 16 bands). (Figure 7)

By means of PCR, no isolates in this group did contain the insertion sequence IS 901 and was therefore determined as *M. avium* ssp. *hominissuis* strain except one isolate which was later confirmed as *M. avium* ssp. *avium.* Among the group of polymorphic patterns 128 different patterns were demonstrated from 137 isolates as shown in the figures 4 and 7.

The reproducibility rate of RFLP, determined by the presence of molecular weight marker in this study was 81.25% (39/48).

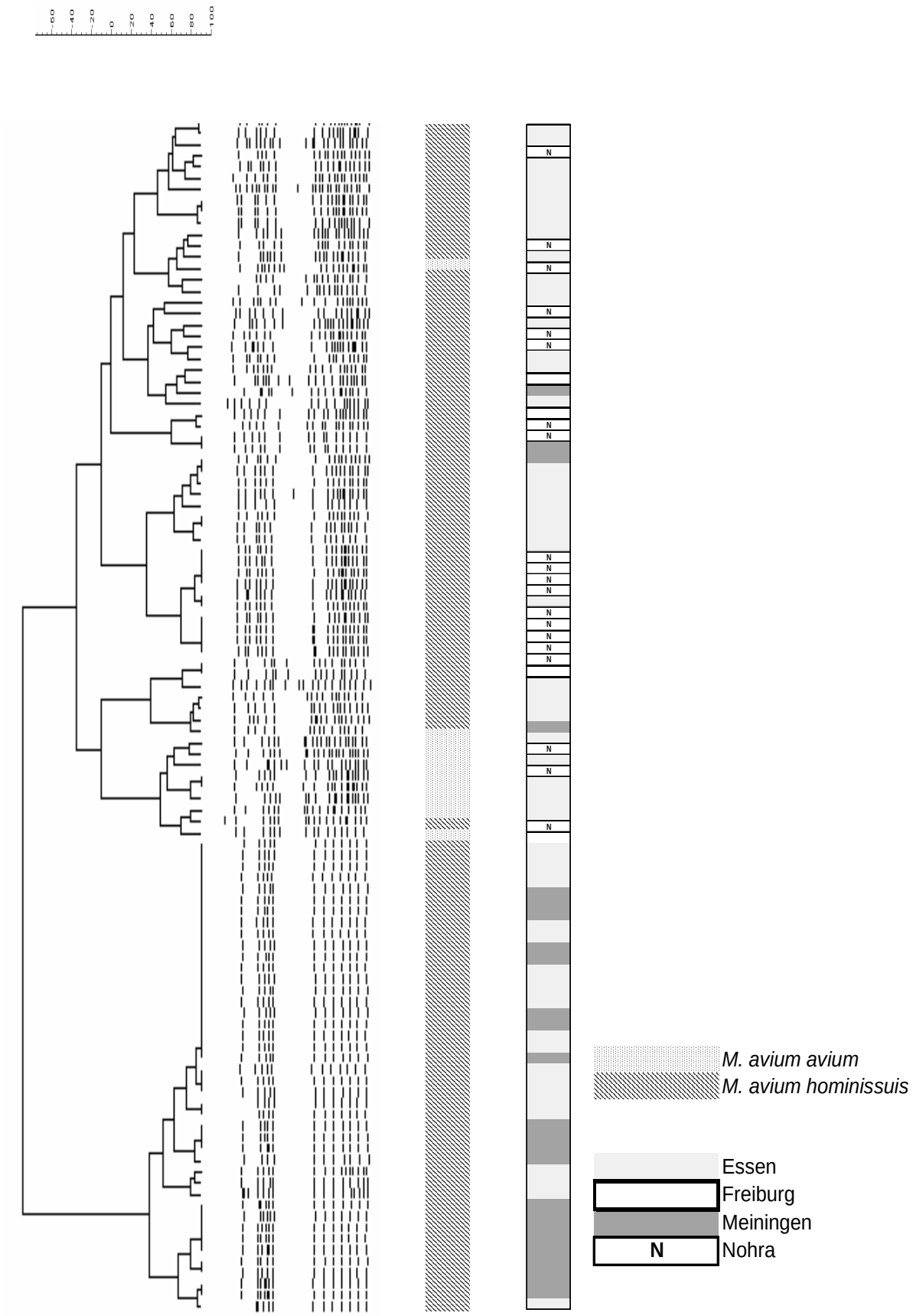

Fig. 2. PFGE Dendrogramme of *M. avium* ssp. *avium* and *M. avium* ssp. *hominissuis* isolated from pigs generated by *Xba* I enzyme.

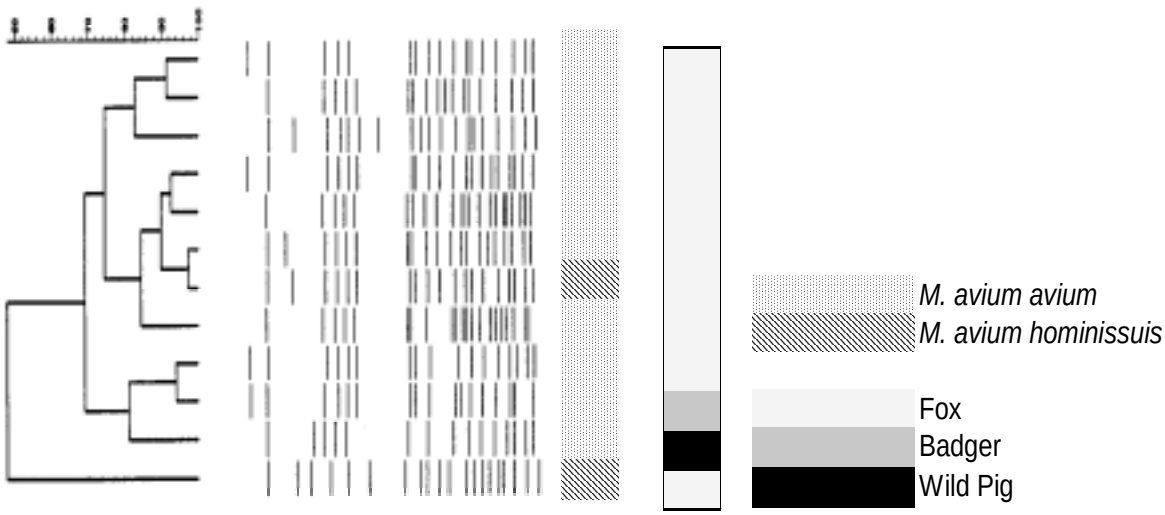

Fig. 3. PFGE dendrogramme of MAIC isolated from wild animals generated by *Xba* I enzyme.

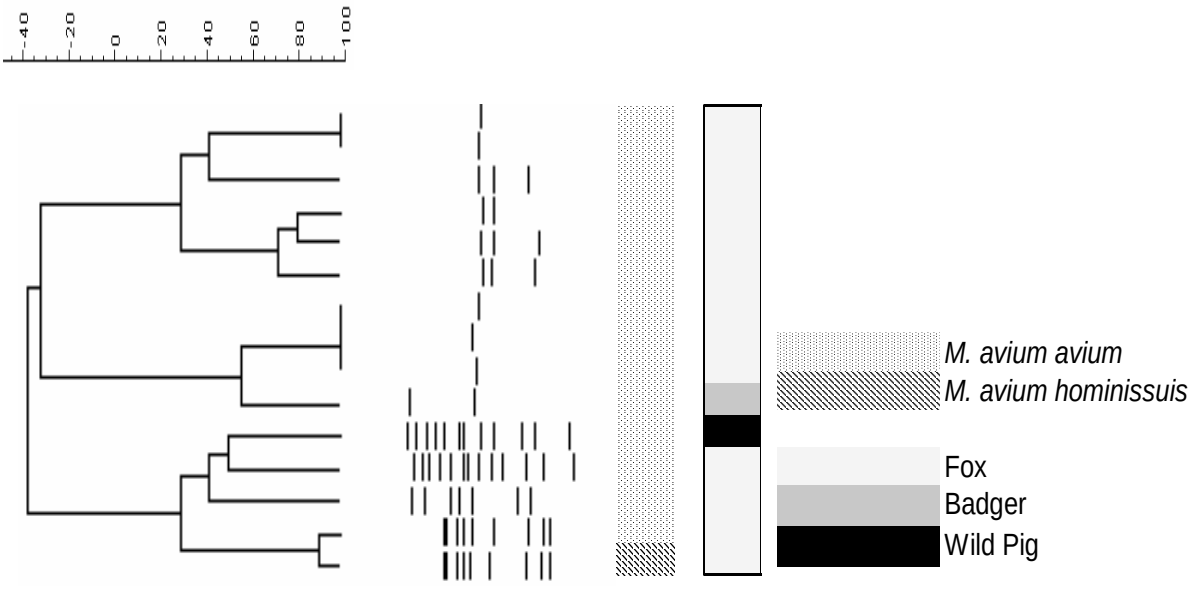

Fig. 4. IS 1245 RFLP dendrogramme of MAIC isolated from wild animals generated by *Pvu* II enzyme.

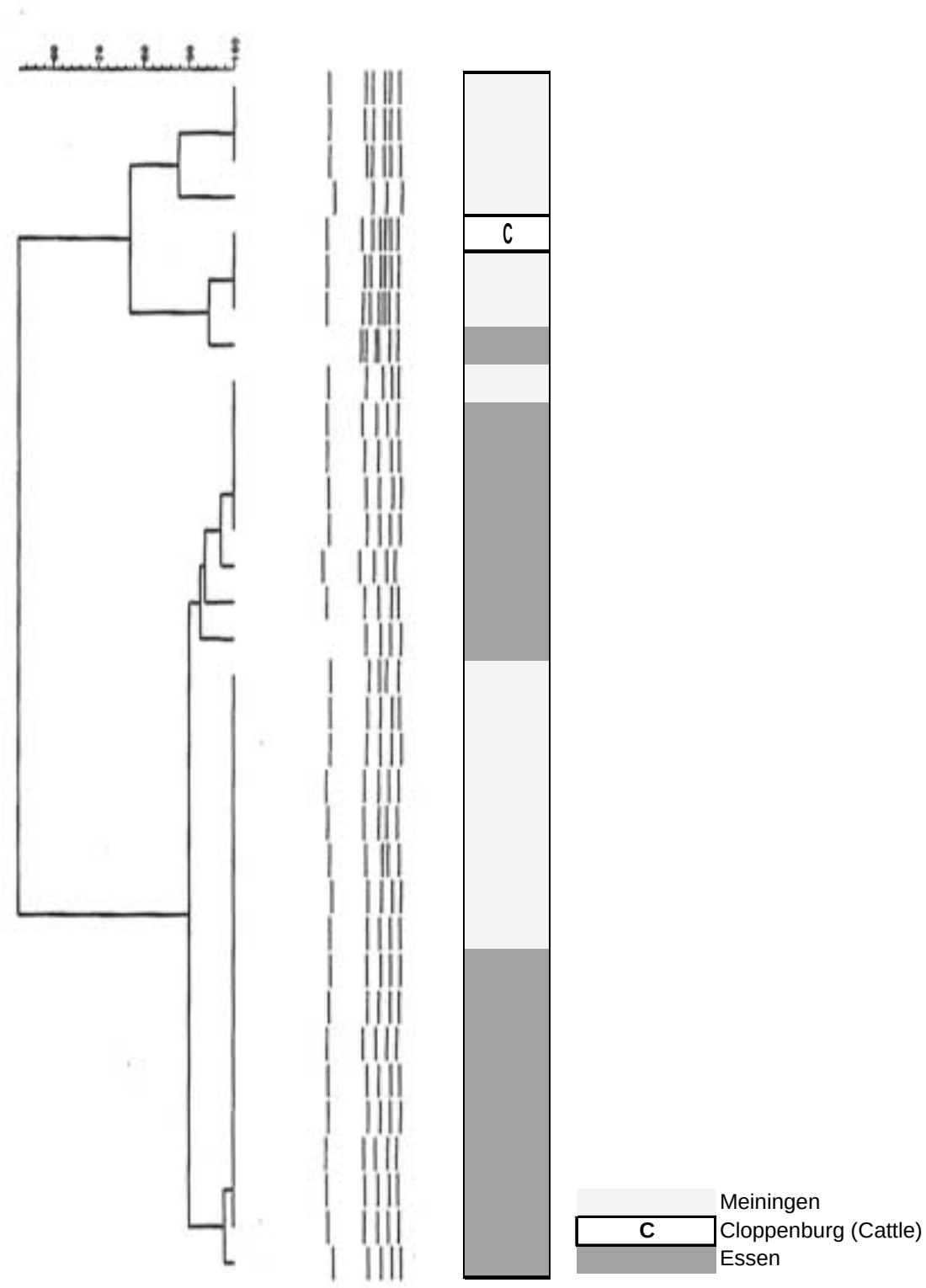

Fig.5. PFGE Dendrogramm of Cloppenburg-like *Mycobacterium avium* ssp. *hominissuis* isolated from pigs groupped by slaughterhouseand cattle from Cloppenburg.

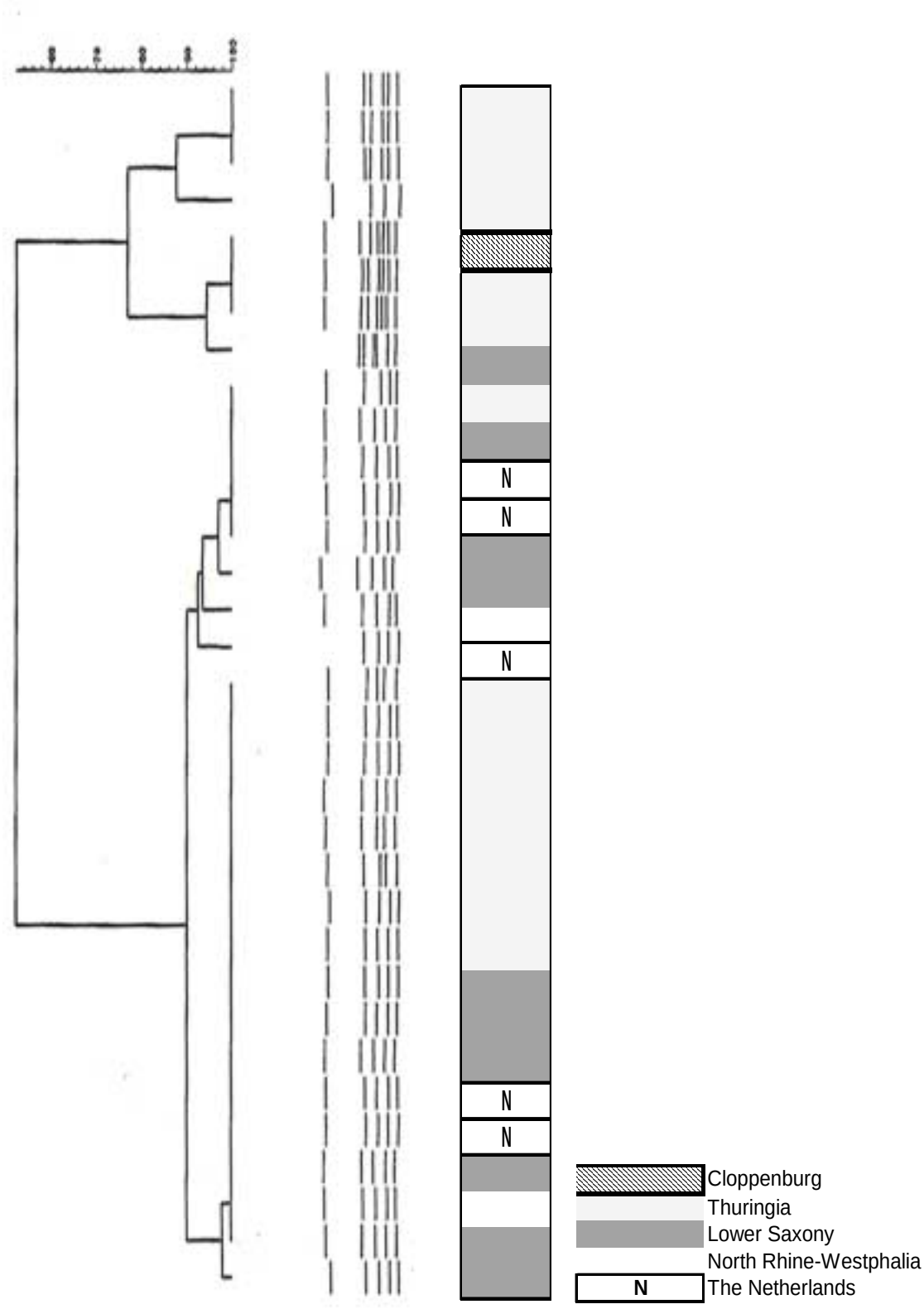

Fig. 6. PFGE Dendrogramme of Cloppenburg-like *Mycobacterium avium* ssp. h*ominissuis* isolated from pigs groupped by geographic origin and cattle from Cloppenburg.

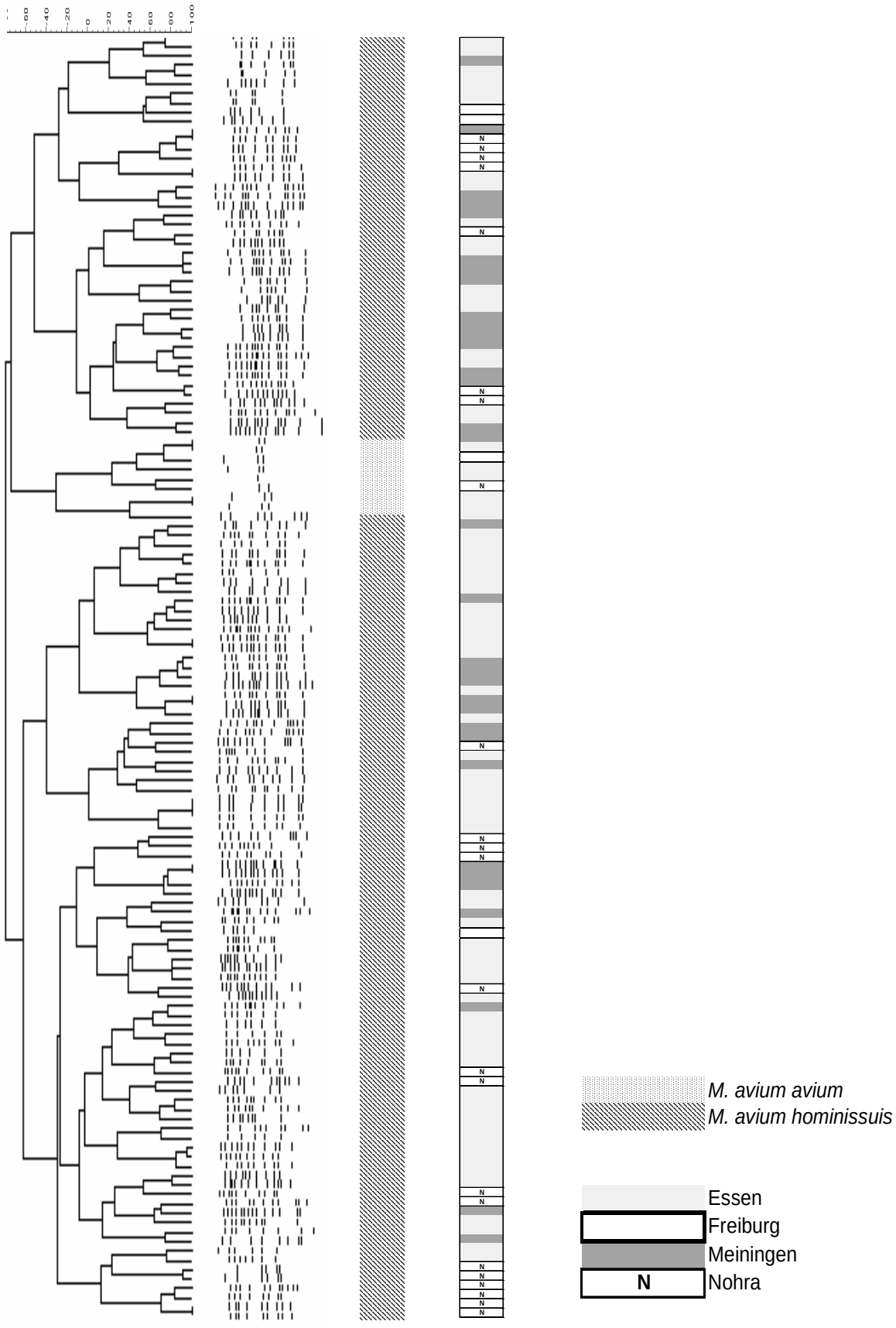

Fig. 7. IS1245 RFLP dendrogramme of *M. avium* ssp. *avium* and *M. avium* ssp. *hominissuis* isolated from pigs generated by *Pvu*II enzyme.