3. Materials and methods

3.1 Sample collection

Lymph nodes from pigs

All lymph nodes were collected immediately after slaughtering and transferred to the laboratory either fresh or frozen at –20°C. All pigs were clinically healthy. Three culture examinations were performed from each lymph node.

390 lymph nodes were collected from 78 randomized pigs in a slaughterhouse of Nohra, Thuringia, Germany, during 30th April 2003 to 22nd July 2003. From each pig 5 lymph nodes were collected (*Lymphonodus mandibularis, Lnn. tracheobronchiales, Lnn. mesenterialis, Lnn. subiliaci and Lnn. poplitei).* None of them showed any typical pathoanatomical lesion of mycobacteriosis.

16232 carcasses were examined during slaughtering process in a slaughterhouse of Essen, North Rhine-Westphalia, Germany. 125 lymph nodes from 88 pigs were collected during 12th – 14th August 2003 (88 *Lnn. mandibulares*, 26 *Lnn*. m*esenteriales* and 11 *Lnn. tracheobronchiales).* All of them showed typical pathoanatomical lesion of mycobacteriosis.

111 lymph nodes were collected from 8 pigs in the slaughterhouse in Freiburg, Baden-Württemberg, Germany, during 9th July - 25 th August 2003. From each animal at least 1 lymph node showed typical pathoanatomical lesion. (15 *Lnn. mandibulares*, 13 *Lnn. mandibulares accessorii*, 15 *Lnn. retropharyngei mediale*s, 15 *Lnn. retropharyngei laterales*,

14 *Lnn. cervicales superficiales*, 13 *Lnn. poplitei*, 13 *Lnn. subiliaci*, 7 *Lnn. jejunales* and 7 *Lnn. tracheobronchiales*).

- 215 lymph nodes were collected from 43 pigs in a slaughterhouse in Meiningen, Thuringia, Germany during 20th May -7th July 2003 (43 *Lnn. retropharygei* mediales, 43 *Lnn. tracheobronchiales,* 43 *Lnn. mesenteriales,* 43 *Lnn. subiliaci* and 43 *Lnn. poplitei)* All pigs were from an "ecological farm" using feed stuffs from ecological plantation, using straw as the bedding material, keeping the pigs in houses combined with out door area and giving 8-9 months time to fatten pigs. None of them showed any typical pathoanatomical lesion of mycobacteriosis.

Mycobacterial isolates from wildlife animals.

152 Lymph nodes from wildlife animals were sent to BfaV (Federal Research Centre for Virus Diseases of Animal) in Jena by TLLV (Thuringia office for food safety and veterinary examination) during 2002-2003 to detect mycobacteria. Total of 22 isolates were cultured and confirmed as the member of MAIC by PCR.

3.2 Bacterioscopy

The Ziehl-Neelsen stain was performed as described by Isenberg (1992).

- Fresh smears of lymph nodes were prepared on objective slide, air dried and heated in an 80°C oven for 20 minutes to inactivate the microbes.

- The entire slide was covered with 10% carbol-fuchsin and heated for two times by passing the flame underneath the slide.

- The slide was cooled down and rinsed with de-ionized water.

- The slide was decolorized for 2 times in 3% acetic-alcohol, 2 minutes each.

- The decolorized slide was counterstained with methylene blue for 2 minutes, rinsed with de-ionized water and air dried.

- The stained slide was examined under light microscope at 1000 times magnification and acid-fast bacilli (AFB) were reported according to recommendation of Centre of Disease Control and Prevention, USA, in 1995 as shown in table.

Table 1. Recommendation to report AFB from tissue stained with ZN.

Number of AFB observed	Report	Degree
None	Negative	-
1-2/300 fields	Number of AFB	±
1-9/100 fields	Average No./100 fields	1+
1-9/10 fields	Average No./10 fields	2+
1-9/ field	Average No./ field	3+
> 9 / field	> 9/ field	4+

AFB = Acid-fast bacilli

3.3 Cultivation

- Lymph nodes were flamed to eliminate bacterial contamination on the surface area.

- 1 gram of lymph node tissue was cut into small pieces and homogenized with 10 ml PBS by a tissue homogenizer.

- Homoginized material was transferred into 50 ml centrifuge tube, an equal volume of NALC-NaOH solution was added. The test tube was incubated for 20 minutes on a shaking plate.

- The suspension was filled up with PBS-Tween 80 to reach 40 ml and centrifuged for 20 minutes, 3500x g.

- The sediment was mixed with 1 ml PBS and inoculated at the dose of 100µl onto 3 culture media: Ogawa- with glycerol, OADC and PACT supplement, Stonebrink- with OADC and PACT supplement and Löwenstein-Jensen with OADC culture media.

- Test tubes were incubated at 37°C under aerobic conditions and examined weekly for the period of 3 months.

3.4 Polymerase chain reaction (PCR)

DNA Isolation

- A single colony from either selective agar was resuspended in 200µl of distilled water and inactivated in 80°C waterbath for 20 minutes in plastic reaction tubes.

- Mycobacterial cells were disrupted by 35 kHz sonication for 10 minutes and transferred to 100°C waterbath for 10 minutes.

- The reaction tube was centrifuged at 12000 x g for 5 minutes and the supernatant was collected for PCR reaction.

PCR amplification and electrophoresis

- 1µl of supernatant was added to a PCR reaction tube and mixed with 0.2 µl of each primer (100 pmol/µl) together with the mixture of 10µl PCR Master mix, 4 µl Q solution and distilled water filled up to 20 µl.

- The complementary copies of template and primers were amplified in a thermocycler as shown in table 2.

- PCR products were separated in 0.8 % agarose gel at 70 volts for 1 hour and visualized under UV light.

Tabel 2. Primers and PCR conditions for the identification of MAIC.

Primers	**Target**	**Thermocycling conditions (c, seconds)**					**Product size**
		1. Denat	Denat.	Anneal.	Exten.	Cycles	(base pairs)
Mycgen (Wilton and Counsins, 1992)	16S rDNA						1030
5'- AGA GTT TGA TCC TGG CTC AG -3'		96	96	60	72	40	
5'- TGC ACA CAG GCC ACA AGG GA -3'		60	15	60	60		
IS 901 (Kunze et al., 1992)	IS 901						1108
5'- GCA ACG GTT GTT GCT TGA AA -3'		96	96	65	72	30	
5'- TGA TAC GGC CGG AAT CGC GT-3'		60	15	60	60		
IS 1245 (Guerrero et al., 1995)	IS 1245						425
5'- GCC GCC GAA ACG ATC TAC -3'		95	95	65	72	25	
5'- AGG TGG CGT CGA GGA AGA -3'		60	20	60	60		
FR 300 (Kunze et al., 1992)	FR 300						300
5'- CAG CCA GCC GAA TGT CAT CC -3'		95	95	65	72	25	
5'- CAA CTC GCG ACA CGT TCA CC -3'		60	20	60	60		

1.Denat. = First denaturation condition, Denat. = Denaturation condition, Anneal. = Annealing condition, Exten. = Extention condition, Cycles = Number of cycles

3.5 Pulsed-field gel electrophoresis (PFGE)

The DNA preparation of PFGE was performed by the method adapted from Hughes et al. (2001).

Cultivation and cell preparation

- The pure culture of mycobacteria was cultivated in 15 ml MB broth with supplement of 1.5 ml OADC and 150 µl of 10% Tween 80 at 37°C until it reached OD 0.2-0.25 at wave length 588 nm.

- In 150 µl of each 1.0 mg/ml cycloserine and 0.1 mg/ml ampicillin were added and incubated at 37°C for one day.

-Mycobacterial cells were centrifuged at 15000 rpm for 20 minutes, resuspended with 1ml TE buffer and kept at –80°C overnight.

Gel block preparation

- To the thawed sample 20 µl of 100 mg/ml lysozyme was added and incubated at 37°C for 1 hour

-500 µl of 1.2% low-melting agarose was mixed with 500 µl of mycobacterial suspension, poured onto gel plug mold and kept in refrigerator for polymerization.

-The gel block was treated with mixture of 1 ml 0.5 M EDTA, 100 µl 10% SDS and 50 µl of 20 mg/ml proteinase K in 56°C waterbath for two days.

-Gel block was briefly washed in distilled water followed for 3 times in TE buffer, for 45 minutes each.

- Bacterial DNA in the gel block was digested with *Xba* I restriction endonuclease in the mixture of 1 µl *Xba* I enzyme (20 unit), 5 µl of 10x restriction buffer recommended by the manufacturer, 0.5 µl of 10 mg/ml BSA and 43.5 µl distilled water at 37°C for 4 hours.

- Digested gel block was loaded onto 1% agarose for PFGE in 300 ml TBE buffer and bacterial DNA was separated in agarose gel by following cycle: 3x14 hours, 5 to 35 seconds logarithmic mode, 150 to 170 volts logarithmic mode and 115° to 130° of electrodes angle logarithmic mode.

- Gel was stained in 100µl/l ethidium bromide for 45 minutes and analyzed by Gel Compar computer programme version 3.0.

3.6 Restriction fragment length polymorphism (RFLP)

The RFLP was adapted from and performed according to the proposal for standardization of van Soolingen et al. (1998).

DNA isolation

- Each pure culture of IS 1245 positive mycobacteria confirmed by PCR was inoculated in 3 tubes of 8 ml MB broth and incubated at 37°C for 1 month.

-Bacterial culture was inactivated in 80°C water bath for 20 minutes and centrifuged at 4000 x g for 30 minutes.

- The supernatant was removed. Bacterial cells from 3 tubes were transferred into one tube, resuspended in 1 ml of 0.1x TE buffer and centrifuged at 4000 x g for 30 minutes.

- The supernatant was removed. Bacterial cells were resuspended in

0.5 ml of 0.1x TE buffer and transferred to 1.5 ml plastic reaction tubes.

- The bacterial suspension was centrifuged at 4000 x g for 30 minutes. The supernatant was removed and the pellet was resuspended with 450 µl of 0.1x TE buffer and kept at –20°C overnight.

-50 µl of 10 mg/ml lysozyme was added to each sample and was incubated at 37°C in a thermoshaker for 3 hours.

- The mixture of 5 µl proteinase K (10 mg/ml) and 70 µl of 10% SDS was added to the sample and incubated in 65°C water bath for 10 minutes.

- 100 µl of 5 M NaCl and 100µl of CTAB/NaCl were added to each sample and incubated at 65°c for 10 minutes.

- The sample was thoroughly mixed with 750 µl 4°C chloroform-isoamylalcohol and centrifuged at 14000 rpm for 5 minutes. The upper part of supernatant (750 µl approximately) was collected in a new plastic reaction tube.

- 750 µl of 4°C isopropanol were added to the sample, gently mixed and kept at –20 °C for 30 minutes.

- Samples were centrifuged at 14000 x g for 15 minutes. Supernatant was removed except the last 20 µl.

- 1 ml of 4°C 70% ethanol was added to the sample and centrifuged at 14000 rpm for 5 minutes. Supernatant was removed.

- To dissolve DNA pellet, 50 µl of distilled water were added and incubated at 37°C in thermomixer for 16 hours.

- The DNA concentration was determined by spectrophotometer at wavelength 260 and 280 nm and calculated by Wartburg-Christian assay.

- Amount of 3 µg DNA was incubated at 37°C overnight in the mixture of 1 µl (10 unit) *Pvu* II enzyme, 2 µl buffer recommended by enzyme manufacturer and distilled water up to 20 µl.

DNA digestion and transfer

- Digested DNA was loaded in 1.2 % agarose gel in 1x TBE buffer and separated by electrophoresis at 110 volts for 3 hours.

- Agarose gel was washed with distilled water, depurinized with 250 mM HCl and denatured in 1.5 M NaCl /0.5 N NaOH for 2 times, 15 minutes each.

- The neutralization of the gel was performed by washing the gel with 0.5 M Tris-HCl/3M NaCl for 2 times, 15 minutes each.

- DNA in the gel was transferred to a hybridization membrane in 2x SSC buffer by vacuum blotter at 100 mbar for 2 hours.

- The membrane was dried in 80°C oven for 2 hours.

DIG-marked gene probe preparation

- 5µl DNA from positive control was amplified with specific primers for IS 1245 (P1: 5'-GCC GCC GAA ACG ATC TAC-3' and P2: 5'-AGG TGG CGT CGA GGA AGA-3') in the

mixture of 10 µl 10x PCR buffer (15 mM $MgCl_2$), 2µl DIG-DNA labeling mix, 0.34µl of each primer (100pmol/µl), 1µl Taq polymerase and 81.3µl distilled water.

- The complementary copies of template and primers were amplified in thermocycler by following conditions:

  - first denaturation, 300 seconds at 94°C

  - following denaturation, 40 seconds at 94°C; annealing 40 seconds at 65°C; extension, 90 seconds at 72°C. These steps were repeated for 35 times.

  - final extension for 300 seconds at 72°C

- DNA was separated in 3% agarose gel electrophoresis in 1x TBE buffer by 40 volts.

- DNA band in agarose gel was cut by scalpel and transferred to a DNAse and RNAse-free plastic tube.

- DNA was melt with NaCl solution and incubated at 55°C for 5 minutes.

- DNA was bound with 6.5µl DIG-DNA-labeling and mixed at room temperature for 5 minutes.

- The mixture was centrifuged at 12000 x g for 5 seconds and the supernatant was removed.

- The pellet was washed by 1 ml washing solution and centrifuged at 12000 x g for 5 seconds.

- The supernatant was removed and the pellet was dried at room temperature.

- The pellet was fully resuspended with 20 µl distilled water for 5 minutes at room temperature and kept at -20°C.

DNA detection

- DNA containing membrane was prehybridized in 50ml hybridization solution. After incubation time of 90 minutes at 68°C, solution was removed.

- DIG-marked gene probe was activated in 100°C waterbath for 5 minutes, cooled down in ice for 3 minutes and added to membrane.

- Membrane was hybridized in DIG-marked gene probe at 68°C overnight.

- Membrane was washed with 50 ml 2x SSC-SDS at room temperature (RT) for 2 times, 5 minutes each; 50 ml 0,1x SSC-SDS at 68°C for 2 times, 15 minutes each and 50ml wash buffer at room temperature for 15 minutes.

- The hybridization solution was replaced with 100 ml blocking solution, the membrane was incubated for 30 minutes at room temperature, replaced with 25 ml 1:5000 anti-DIG-AP-conjugate in blocking solution and was incubated again at RT for 60 minutes.

- The membrane was washed for 2 times with 100 ml washing buffer and was incubated in 20 ml detection buffer for 5 minutes.

- After the removal of detection buffer, membrane was incubated in 5 ml of 1:100 CSPD substrate in detection buffer for 5 minutes.

- The membrane was packed in transparent foil and incubated at 37°C for 15 minutes to enhance the signal.

- Chemiluminescent signal was detect in the dark by Hyperfilm ECL, exposion time 15-20 minutes. The hyperfilm was developed for 1 minute and fixed for 5 minutes.

- DNA bands were analyzed by Gel Compar computer programme version 3.0.