

Application of PCR-SSCP for molecular epidemiological studies on the exposure of farm children to bacteria in environmental dust

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Abstract

The environmental exposure of farm children to microorganisms in dust has become a focus of interest, since microbial exposure on farms has been related to a reduced prevalence of asthma and atopic diseases in children. Previous studies almost exclusively focused on the determination of microbial counts using conventional culturing or the determination of microbial compounds i.e. endotoxins. In this study PCR-SSCP (single-strand conformation polymorphism) was modified for characterising bacterial communities in environmental dusts and their sensitivity and reproducibility was validated. A fivefold repeated PCR-SSCP analyses of a well homogenised mattress dust, cow-shed dust, swine-shed dust, chicken-shed dust and a horse-shed dust sample, respectively, showed similarities, based on Pearson correlations, ranging from 89.7% to 95.2%. The reproducibility of day to day variations (five days) and gel to gel variations (five gels) was also around 90%. The detection limit of *Escherichia coli* was 7×10^1 cfu g⁻¹ whereas *Listeria monocytogenes* and *Bacillus licheniformis* containing 30% spores showed visible bands at 7×10^2 cfu g⁻¹. Application of this method to dust samples of 37 sheds and 63 children's mattresses showed that distinct farm environment dusts reflected different SSCP profiles. However, digital analysis of the gels showed that some bands in the profiles of shed- and mattress dusts were found at the same position in the gels. By excision, cloning, sequencing and phylogenetic analyses, these bands were identified as *Corynebacterium tuberculoearicum*, *Corynebacterium mucifaciens*, *Staphylococcus epidermidis*, *Acinetobacter lwoffii*, *Brevibacterium iodinum*, *Brevibacterium linens* and *Arthrobacter* spp, respectively. These results may reflect transfer of microorganisms from animal sheds to mattresses. In conclusion this study demonstrates that PCR-SSCP is a promising method with sensitive detection limits and moderate sample variances to be applied for epidemiological studies characterizing the exposure of farmers using environmental dust.

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1. Introduction

During recent decades, there has been an increasing interest in the environmental exposure of farmers to dust microorganisms, but results obtained from such studies are still controversial. Several studies with focus on occupational medicine have demonstrated an association of human exposure to airborne bacteria or their components, particularly endotoxins, in animal sheds with respiratory diseases such as organic dust

toxic syndrome, chronic obstructive pulmonary diseases and asthma-like syndrome (Radon, 2006; Radon et al., 2004; Singh and Schwartz, 2005; Linacker and Smedley, 2002). In contrast, farm children who were exposed to animal sheds and farm animals in early life consistently showed lower prevalences of hay fever, atopic sensitization, and with some limitation asthma, than children of the same age not exposed to such an environment which may be induced by the microbial flora (Debarry et al., 2007; Van Strien et al., 2004; Braun-Fahrlander et al., 2002; Riedler et al., 2001; von Mutius et al., 2001). The effect seems to be sustained until adulthood (Leynaert et al., 2006; Radon et al., 2006).

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A strong limitation of the hitherto existing studies is that they were all based on the analyses of microbial counts to quantify microbial exposures of humans using conventional culturing and measurement of microbial compounds such as endotoxin and *N*-acetyl-muramic acid (Debarry et al., 2007; Lee et al., 2006; Van Strien et al., 2004; Albrecht et al., 2003; Radon et al., 2002; Braun-Fahrlander et al., 2002; von Mutius et al., 2001; Mackiewicz, 1998). Both methods, however, do not reflect the total microbial diversity. Traditional microbiological techniques based on cultivation are limited in their potential to detect environmental microorganisms, since more than 90% of all microorganisms in nature cannot be isolated with common growth substrates and culture conditions (Amann et al., 1995). The determination of microbial compounds is inappropriate because only sub-populations are represented, such as Gram-negative bacteria in the case of endotoxin, which is only a part of the Gram-negative cell wall (Waser et al., 2004).

In addition to cultivation, molecular methods based on PCR-amplified target gene sequences from directly extracted environmental nucleic acids allow detection and characterization of microorganisms. The most suitable prokaryotic gene to study in this context is the 16S rDNA which encodes for ribosomal RNA, present in all living organisms. Genetic profiling methods, i.e., DGGE (denaturing gradient gel electrophoresis) (Muyzer et al., 1993) and SSCP (single-strand conformation polymorphism) (Schwieger and Tebbe, 1998; Zumstein et al., 2000) open the possibility to visualise the diversity of such amplified genes from environmental DNA and thereby allow to compare the bacterial diversity from different samples independent of or prior to their characterisation by DNA-sequencing. However, in some instances it has been reported that the reproducibility of those methods may be poor (Hong et al., 2007; Nunan et al., 2005) and validations are thus highly desirable before such tools are applied in health-related studies. Hori et al. (2006) noted higher resolution when comparing SSCP as developed by Schwieger and Tebbe (1998) to DGGE, which was also confirmed in our own laboratory (data not shown). Therefore this study aimed at establishing PCR-SSCP to characterize bacterial communities in farming environmental dusts. Since this method should be applied for large-scale epidemiological studies this study secondly aimed at validating this method concerning to reproducibility and sensitivity.

2. Materials and methods

2.1. Filed samples

During spring 2006, dust samples of 37 sheds and 63 mattresses from 26 farming families were collected at different locations in Bavaria (Southern Germany). Dust specimens from the sheds were taken from sediments using brushes and bags previously treated with 70% ethanol. The whole area of a mattress was vacuumed for 3 min and dust was collected in nylon socks according to a standard protocol (Schram-Bijkerk et al., 2005).

A total of 2.85 ml phosphate-buffered saline was added to 150 mg of dust and shaken at 350 rpm (GFL 3005, Germany) for 30 min. Larger dust particles were collected by low-speed centrifugation (200 ×g, 30 min). The supernatants were

transferred into 1.5 ml tubes and centrifuged at 10,000 ×g for 10 min to obtain a sediment with bacterial cells which were further subjected to DNA extraction.

2.2. Extraction of bacterial DNA

The DNA extraction was performed according to the protocol of Somerville et al. (2005) with minor modifications. After chloroform-isoamylalcohol (24:1) treatment 600 µl of the aqueous phase (upper phase) was transferred in a 1.5 ml tube, 2 µl glycogen (Invitrogen, Germany) and 600 µl icecold isopropanol (100%) were added to the tube and mixed, followed by a centrifugation in a microcentrifuge (VWR, Germany) at 16,000 ×g for 10 min at room temperature. The pellet was washed in 600 µl of 70% (vol./ vol.) ethanol and centrifuged at 16,000 ×g for 2 min in a microcentrifuge. Ethanol was removed and the resulting genomic DNA pellet was resuspended in 30 µl nuclease-free water.

2.3. Modifications of single-strand conformation polymorphism (SSCP)

PCR-SSCP was used as developed by Schwieger and Tebbe (1998) and described in detail by Dohrmann and Tebbe (2004). The method includes a conversion of the double stranded PCR products into single strand by enzymatic removal of one DNA strand, generated during PCR using a phosphorylated primer. For profiling bacterial communities in farming dusts, some modifications were necessary. PCR was performed in a Trio-Thermoblock (Biometra, Germany) using primers Com1 and Com2-Ph as described before (Schwieger and Tebbe, 1998). However, here we used 1.25 U of Hot Star *Taq* polymerase (QIAGEN, Germany) with corresponding 1xPCR buffer comprising 1.25 mM MgCl₂. The PCR products were purified using the QIAquick PCR Purification Kit (QIAGEN, Germany) as described by Dohrmann and Tebbe (2004). Afterwards, DNA was quantified with Nanodrop ND-100 spectrometer (Peglab, Germany), a DNA-solution of 26 µl containing 400 ng DNA was prepared and 14 µl with 5 U lambda exonuclease in 1 × exonuclease buffer (New England Biolabs, Germany) was added. Double-stranded PCR products were digested for 45 min at 37 °C as described by Dohrmann and Tebbe (2004). The digestion was stopped using the MinElute PCR Purification Kit (QIAGEN, Germany) and columns were eluted with 10 µl of the supplied buffer; afterwards, 8 µl of SSCP loading buffer was added. The purified DNA-single strands were denatured for 2 min at 95 °C and immediately cooled on ice directly before 10 µl of sample were loaded into each well of a native (non-denaturing), temperature controlled, vertical polyacrylamide gel for SSCP electrophoresis.

In order to find the most suitable electrophoretic conditions for SSCP, single-stranded DNA of 10 phylogenetically different bacteria including *Escherichia coli* DSM 1103, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 7644, *Clostridium perfringens* DSM 756, *Enterococcus faecalis* DSM 20478, *Bacillus licheniformis*, *Enterococcus faecium* DSM 10477, *Acinetobacter lwoffii*, *Lactobacillus plantarum* WS

1033, *Lactobacillus acidophilus* WS 1617, respectively, were analysed. The samples were electrophoresed in 30 to 40% MDE-gels, (MDE-solution, Cambrex, Belgium) gel with 1× TBE buffer. They were cast in a PerfectBlue Dual Gel System Twin L (Peqlab, Germany) using 0.4-mm spacers and combs. Based on the DNA concentrations of the PCR products, each lane of an SSCP-gel was loaded with the same amount of total single-stranded DNA to increase comparability of the SSCP profiles on a gel with each other. Electrophoresis was performed for 13 to 18 h at 400 V and 20 °C. The gels were silver stained according to Bassam et al. (1991) and dried at room temperature.

Similarities of SSCP profiles of dust bacterial communities were analysed with the GelCompar II software (version 4.6; Applied Maths, Belgium). Individual SSCP patterns were normalised by using a standard containing a mixture of DNA-single strands of *L. monocytogenes*, *B. licheniformis*, *Mycobacterium vaccae*, *Bacillus subtilis*, *L. plantarum* and *E. faecalis*, respectively.

Similarity matrix calculations were based on Pearson correlation coefficients as they could be calculated with GelCompar II. The clustering method for dendrograms of similar groups was UPGMA (unweighted pair group method using arithmetic averages).

In order to determine their DNA sequence, selected bands from SSCP profiles were carefully cut out from the gels by using a sterile, DNA-free scalpel. The DNA was recovered by the crush and soak procedure as described by Peters et al. (2000), re-amplified by PCR with the primers Com1 and Com2-Ph (Schwieger and Tebbe, 1998), purified using the NucleoSpinExtract-Kit (Macherey-Nagel, Germany), ligated into the pGEM-T-vector system (Promega, Germany) and transformed in *E. coli* JM 109 (Promega, Germany) according to a protocol provided by the manufacturer. Clones were cultivated overnight in Luria-Bertania (LB) broth containing ampicillin (final concentration 100 mg l⁻¹). Transformed cells were selected by blue-white screening. The plasmids were extracted with the NucleoSpinPlasmid-Kit (Macherey-Nagel, Germany) and sequenced by cycle sequencing, using the ABI 373048 capillary sequencer with 50 cm capillary length and the Big Dye[®] Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Germany). The primer for sequencing was M13-21 (5'-3') TGTAACGACGGCCAGT (TIB Molbiol, Germany). The DNA sequences were analysed for their closest relatives using the Ribosomal Database Project II (http://www.rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) for phylogenetic analysis.

2.4. Validation of the PCR-SSCP-procedure for dust analysis

To determine the sensitivity of detection, twenty-one dust specimens of 150 mg each were gamma irradiated (26 kGy for 22 h) in order to degrade the DNA naturally occurring in dust. Seven dilutions of suspensions of *L. monocytogenes* (Gram-positive), of *B. licheniformis* (spore forming bacterium) and *E. coli* (Gram-negative) in phosphate-buffered saline ranging from 10⁸ to 10² cfu ml⁻¹ were prepared, and 10 µl of each dilution was added to the 150 mg mattress dust specimens. The

spore content of the *B. licheniformis*-suspension was determined by quantitative microscopic examination where 30% spores were found.

To validate the reproducibility of the PCR-SSCP technique, a mattress dust, a cow-shed dust, a swine-shed dust, a horse-shed dust and a chicken-shed dust, respectively, were well homogenised, and the DNA of each sample was fivefold extracted and processed for PCR-SSCP. To analyse the day to day variation, DNA of one mattress dust was fivefold extracted every day over a period of 5 days and PCR-SSCP was performed. Gel to gel variation was studied by loading single-stranded DNA of one mattress on 5 SSCP-gels at the same concentration (400 ng) to compare the SSCP profiles among the gels. All similarity profiles were calculated as Pearson correlation coefficients (%) using the GelCompare II software.

2.5. Application

To demonstrate the suitability of PCR-SSCP to characterise different microbial exposures of humans in more detail, dust samples of cow-sheds (*n*:24), swine-sheds (*n*:10), chicken-sheds (*n*:2) and horse-sheds (*n*:1) as well as of children's mattresses (*n*:63) of 26 farms were analysed. Densitometric curve analyses using the GelCompare II software was used to analyse similarities of the SSCP profiles. Conspicuous bands were excised from the gel, cloned and sequenced as described above.

3. Results and discussion

3.1. Adaptation of SSCP-electrophoresis conditions

In general, resolution for discriminating different PCR products with SSCP was best at 16 h running time and 31.7%

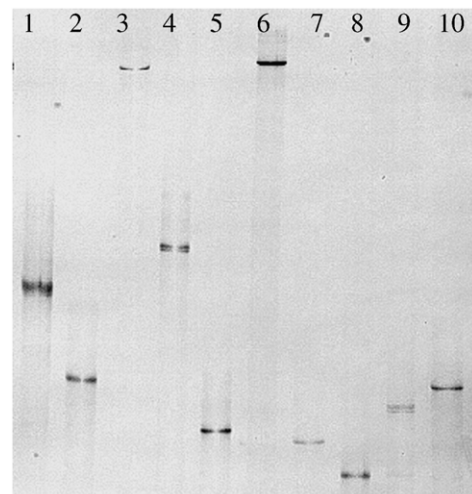


Fig. 1. SSCP profiles obtained from different bacterial species after PCR amplification a partial region of the DNA encoding the 16S rRNA gene. The following species were analysed: *Escherichia coli* DSM 1103 (lane 1), *Staphylococcus aureus* ATCC 25923 (lane 2), *Listeria monocytogenes* ATCC 7644 (lane 3), *Clostridium perfringens* DSM 756 (lane 4), *Enterococcus faecalis* DSM 20478 (lane 5), *Bacillus licheniformis* (lane 6), *Enterococcus faecium* DSM 10477 (lane 7), *Acinetobacter lwoffii* (lane 8), *Lactobacillus plantarum* WS 1033 (lane 9), *Lactobacillus acidophilus* WS 1617 (lane 10).

MDE concentration (data not shown). Using these conditions, PCR products of 10 phylogenetically different bacteria (phylotypes) expected in farm environments led to single- or double bands in different positions (Fig. 1). The majority of PCR products in our investigation amplified from the pure cultures were found to be single-bands. These products were different in electrophoretic mobility, most probably due to sequence differences causing different conformations under non-denaturing conditions (Dohrmann and Tebbe, 2004). However, single sequences can also cause double bands due to conformational isomers. Other double bands may have resulted from the presence of more than one operon with intra-operon sequence variations even within one phylotype (Schmalenberger et al., 2001).

3.2. Validation of the PCR-SSCP-procedure for dust analysis

Validations of the SSCP method were regarded as indispensable before applying the technique in field studies on health-related topics. Reproducibility of gel-based genetic profiling techniques, i.e., DGGE or SSCP has previously been questioned (Hong et al., 2007; Nunan et al., 2005), but actual validations are still missing. Therefore, the present study tested both sensitivity and reproducibility of SSCP-profiling of partial 16S rRNA gene sequences.

The detection limit for the Gram-negative bacterium *E. coli* was 70 cfu g⁻¹, whereas a total of 700 cfu g⁻¹ dust was necessary for the detection of *B. licheniformis* containing 30%

spores and the Gram-positive bacterium *L. monocytogenes*, respectively. Compared to Gram-negative bacteria, the substantially higher detection limit for Gram-positive bacteria was possibly caused by an incomplete cell lysis because of the more resistant Gram-positive cell wall (Von Wintzingerode et al., 1997). Consequently, DNA of Gram-positive bacteria may be underrepresented in genetic profiles. On the other hand more rigorous cell lysis conditions may cause highly fragmented nucleic acids from Gram-negative cells (Von Wintzingerode et al., 1997) and thus would also induce bias. The sensitivity of the detection of spore forming bacteria might be influenced by their structure. It is obvious that bacterial spores are more difficult to lyse than vegetative cells as spores possess an extremely resistant outer cortex which may hamper release of DNA (Popham and Setlow, 1993; Von Wintzingerode et al., 1997).

Still this method provides advantages over conventional culturing, as it can detect non cultivable bacteria. Conventional culturing definitively does not reflect the whole range of microbial diversity (Wagner et al., 1993). Furthermore, the SSCP method in this study was as sensitive as the culturing method without a prior enrichment procedure (data not shown). This is an important advantage, since, e.g., mattress dust usually is limited to about 150 mg per mattress.

A fivefold repeated analyses of a well homogenised cow-, swine-, chicken- and horse-shed dust sample, respectively, showed highly similar profiles for each replicate (Fig. 2). The average similarity calculated as Pearson correlation coefficients

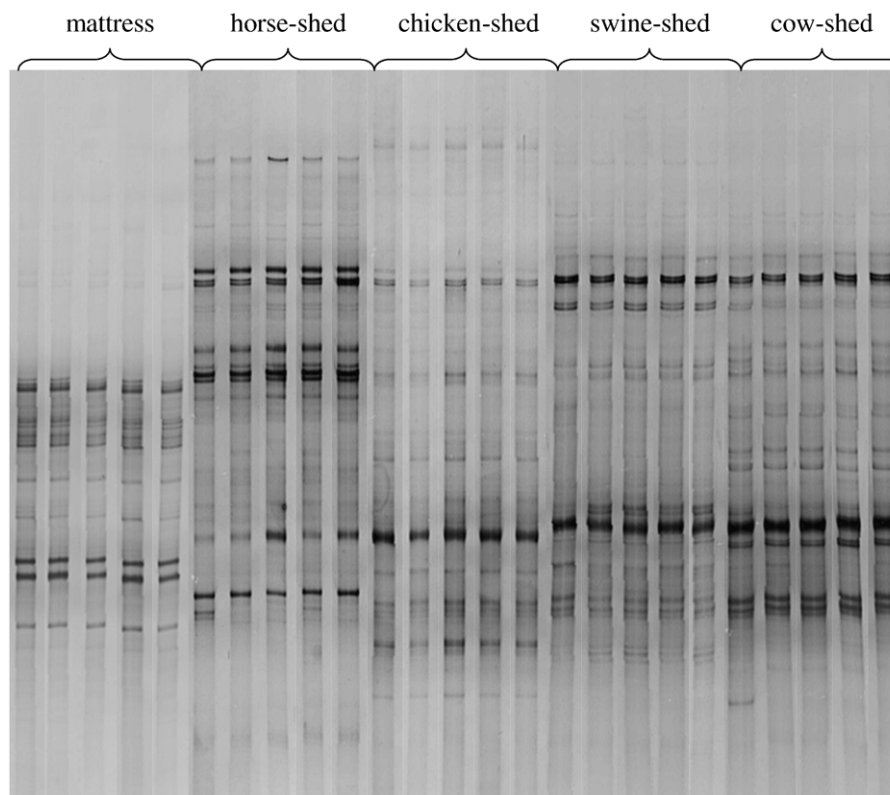


Fig. 2. SSCP profiles from one mattress dust, one horse-shed-, one chicken-shed-, one swine-shed- and one cow-shed-dust. DNA of all dusts were extracted in quintuples and processed for PCR-SSCP.

Table 1

Similarities of SSCP profiles from one mattress dust, one horse-shed dust, one chicken-shed dust, one swine-shed dust and one cow-shed dust calculated as Pearson correlation coefficients (%)

Dust types	Similarity (%)
Mattress dust	95.2 (SD=2.6)
Horse-shed dust	92.9 (SD=4.9)
Chicken-shed dust	89.7 (SD=6.2)
Swine-shed dust	92.9 (SD=3.0)
Cow-shed dust	90.4 (SD=6.2)

DNA of all dusts were extracted in quintuples and processed for PCR-SSCP. Each sample of one dust type was compared with the other samples of the same dust type. Data are shown as average similarities for each dust type.

was above 89% when comparing the five replicate samples from the same dust type with each other (Table 1). Similar high reproducibility values were obtained when one mattress dust was processed for DNA extraction and SSCP analyses over a five day period and profiles of each day were compared, showing 87.2% similarity on average (Table 2). Comparing one mattress dust SSCP-profile on five different gels, the reproducibility was slightly lower but again close to 90% on average (Table 3).

Several studies revealed that the limitation of PCR-based methods is mostly related to inefficient lysis of the bacterial cells and incomplete DNA release (Farrelly et al., 1995; Suzuki and Giovannoni, 1996). Dust is an inhomogenic substrate containing various biotic and abiotic components. Inorganic particles or organic matter affect lysis efficiency and may interfere with subsequent DNA purification and enzymatic steps (Von Wintzingerode et al., 1997). In this study the similarity between the experiments was consistently around 90%. In other words inter-experiment variation was about 10% which is a moderate limit. This low variability makes SSCP an ideal tool to compare larger numbers of samples for environmental exposure studies. But it has to be considered that these reproducibility results are shown for a special dust matrix which does not mean that the same bacterial flora might be fully detected in another dust matrix. To get further evidence for the reproducibility of the present method, additional experiments will be necessary. A possible experiment should e.g. be using the bacterial flora of mattress dust spiked on DNA-free dusts of a cow-shed, swine-shed, chicken-shed and horse-shed to study whether all species of the mattress dust flora are detectable in the other dust matrices as well or if some of the species get lost by matrix effects.

Table 2

Similarities of SSCP profiles of one mattress dust processed by DNA extraction and SSCP analysis on five different days

	Day 1	Day 2	Day 3	Day 4	Day 5
Day 1	100				
Day 2	78.7	100			
Day 3	92.2	89.7	100		
Day 4	92.0	88.0	93.2	100	
Day 5	86.3	81.2	86.5	84.6	100

Data are shown as Pearson correlation coefficients (%).

Table 3

Similarities of SSCP profiles from DNA of one mattress dust processed for SSCP analysis and loaded on five different gels

	Gel 1	Gel 2	Gel 3	Gel 4	Gel 5
Gel 1	100				
Gel 2	84.5	100			
Gel 3	91.7	95.8	100		
Gel 4	80.6	75.2	92.3	100	
Gel 5	87.3	90.2	92.1	73.2	100

Data are shown as Pearson correlation coefficients (%).

3.3. Application

To demonstrate different microbial exposures on a farm, dust samples of cow-sheds ($n=24$), swine-sheds ($n=10$), chicken-sheds ($n=2$), horse-sheds ($n=1$) and children's mattresses ($n=63$) of 26 farms were analysed by PCR-SSCP. Settled dust was used since previous studies revealed that it can pass into the airborne dust fraction when activities inside the stables occur: Schierl et al. (2007) found that the endotoxin concentrations in settled dust followed the same pattern of median endotoxin concentrations, and minimal and maximal values as the inhalable endotoxin fraction in airborne dust for all animal species. According to Makino and Cheun (2003) microorganisms are dispersed in the air in small droplets comparable to aerosol, spore or dust, but most of them are sediment within short time. In the context of large-scale epidemiological bacterial exposure studies, settled dust can be sampled more easily, without elaborate technical equipment and in a more cost-effective way than dispersed dust. With respect to bacterial compositions, airborne dust samples of animal sheds show substantially higher variations over time than samples of settled dust (Schierl et al., 2007). Furthermore most previous epidemiological studies were performed on settled dust, rendering SSCP analyses of settled dust sample more comparable.

Fig. 3 representatively demonstrates 2 of the 26 farms where bands related to specific environments can be discerned. As already demonstrated in Fig. 2, distinct farm environment dusts reflected different SSCP profiles which consisted of 15 to 20 distinguishable bands for each sample, indicating the high potential of this technique to detect different bacteria from environmental dust. Exposure patterns of dust are affected by animal species, housing conditions, feeding management, seasonal climate conditions and diurnal activities (Schierl et al., 2007). In animal houses the main major contributors to bacterial contaminated organic dusts are animal faeces and bacteria-contaminated plant materials (Radon et al., 2002). Examples are silage, cattle and pig manure slurry which all contain a highly diverse bacterial population (Pahlow et al., 2003; Hobson and Stewart, 1997; Snell-Castro et al., 2005). Profiles of farm child's mattress dusts can probably be affected by the human skin containing a high distinct bacterial flora (Dekio et al., 2005), the child's personal cleanliness (Waser et al., 2004), or by children allowing their pets to stay in bed (Waser et al., 2004). Furthermore the outdoor environment might influence the bacterial community in children's mattress

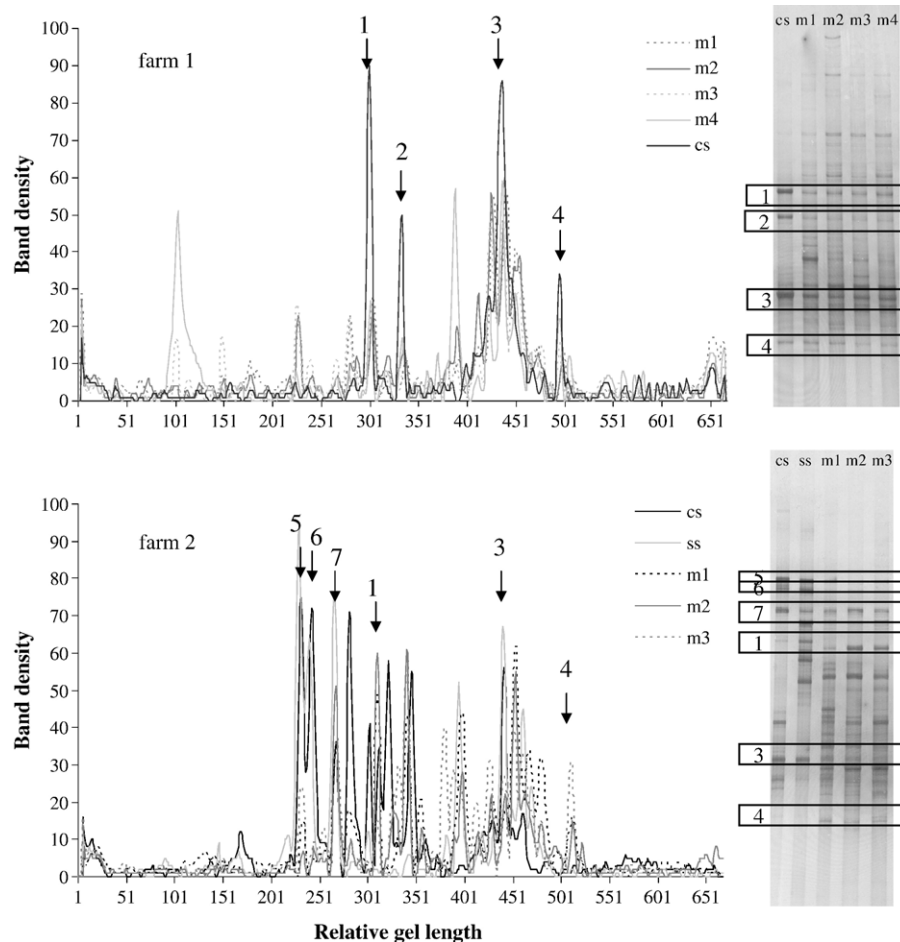


Fig. 3. Representative densitometric curve analysis (a) and SSCP-gel (b) from environmental dusts of 2 farms. Cs=cow-shed, ss=swine-shed, m=mattress. 1=*Corynebacterium tuberculostrictum*, 2=*Corynebacterium mucifaciens*, 3=*Staphylococcus epidermidis*, 4=*Acinetobacter lwoffii*, 5=*Brevibacterium iodinum*, 6=*Brevibacterium linens*, 7=*Arthrobacter* spp.

dust by helping with harvesting, staying in barns and by playing in the sheds. Children might probably carry dust loaded with bacteria on clothes and with their hair into the indoor environment (Waser et al., 2004). A possible transfer of bacteria is shown in Fig. 3, where specific bands present conspicuous in animal shed dust were also found visible in mattresses at the same position in the gel. This finding is in line with Waser et al. (2004) who found a dose-dependent association between the child's activity on the farm and indoor home endotoxin levels. It is likely that children carry dust loaded with bacteria with their hair, clothes and skin from the sheds to the indoor environment.

Using PCR-SSCP, cloning and sequencing of the bands occurring at the same position opens the possibility to identify the bacterial species, which might be transferred from the sheds to the home environment—in this case mattress dust. By excision, cloning, sequencing and phylogenetic analyses of the bands, bacteria from sheds and mattresses were identified as *Corynebacterium tuberculostrictum*, *Corynebacterium mucifaciens*, *Staphylococcus epidermidis*, *A. lwoffii*, *Brevibacterium iodinum*, *Brevibacterium linens* and *Arthrobacter* spp. (Fig. 3). *A. lwoffii* isolated from cow-sheds was already shown to have an asthma preventive potential in a mouse model (Debarry et al., 2007). However, not all of these species were

detected on all farms but the occurrence of bands having the same position in the sheds as well as in the mattresses was observed for each farm. This finding opens the possibility to use PCR-SSCP performed for mattress dust samples in large-scale epidemiological studies on bacterial exposure of farm children. Additionally mattress dust samples can be taken from the control groups like non-farming children as well.

4. Conclusions

In conclusion, this study demonstrates the applicability of PCR-SSCP for assessing the diversity of bacteria found in dust samples from farm environments. By selection of specific SSCP-bands, cloning and sequencing the method allows the assignment of bacteria to closest relatives, as they can be found in public databases like the Ribosomal Database Project, Genbank or the EMBL database. PCR-SSCP therefore opens new possibilities to explore the bacterial exposure of farmers and their children much more precise than the hitherto applied methods. In combination with real-time PCR targeting the species of interest, the method may become even more powerful in the future. The health-related effect of the farm specific bacteria on asthma and allergies could be tested in *in vivo*

exposure experiments. Since a recent study (Schram-Bijkerk et al., 2005) revealed that mould components might offer some protection against atopic wheeze in children, the application of PCR-SSCP to fungi and their spores in dust might be interesting for future studies as well.

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