PCR-based detection of non-indigenous microorganisms in 'pristine' environments - Review article

Gillian C. Baker, Lemese Ah Tow, Don A. Cowan*

Institute for Microbial Biotechnology and Metagenomics, University of the Western Cape, Private Bag X17, Bellville 7535, Cape Town, South Africa

Abstract

PCR-based technologies are widely employed for the detection of specific microorganisms, and may be applied to the identification of non-indigenous microorganisms in 'pristine' environments. For 'pristine' environments such as those found on the Antarctic continent, the application of these methods to the assessment of environmental contamination from human activities must be treated with caution. Issues such as the possibility of non-human dispersal of organisms, stability and survival of non-indigenous organisms in vivo, the sensitivity, reproducibility and specificity of the PCR process (and particularly primer design) and the sampling regime employed must all be considered in detail. We conclude that despite these limitations, PCR and related technologies offer enormous scope for assessment of both natural and non-indigenous microbial distributions.

Keywords: Antarctica; Endemism; Human commensals; Primer design; Psychrophile

1. Antarctica

The Antarctic continent has been geographically isolated from the rest of the world since its separation from Gondwanaland more than 10 million years ago (Vincent, 2000). Some Antarctic habitats have remained isolated for hundreds to millions of years and are considered to be some of the most pristine ecosystems left on earth (Vincent, 2000). In the past 50 years there has been a rapid increase in the numbers of human visitors to Antarctica (Upton et al., 1997; Palmgren et al., 2000) and in recent years it has become a tourist destination with thousands of visitors per year (Enzenbacher, 1992).

The impact of human activities on Antartica's supposedly pristine habitats has become an international concern (Upton et al., 1997). Anxiety over habitat destruction through mining and contamination of the Antarctic ecosystem with hydrocarbons led to the signing of an international treaty on environmental protection in 1991. The extent of chemical pollution in Antarctica has been well documented (Delille, 2000; Delille and Delille, 2000b), as has the importation of some non-indigenous organisms, such as insects (Upton et al., 1997; Palmgren et

al., 2000). However, the extent of biological contamination of Antarctic microbial ecosystems by humans themselves is poorly understood.

Provisions of the 1991 Antarctic treaty has gone some way to limit the direct impact of human-derived wastes, and thereby their microbial populations. However, it is virtually impossible, without overly stringent protocols, to prevent transport and dispersal of non-indigenous microorganisms in the vicinity of tourist sites, research stations and field sites.

2. Human microbiota

There is a huge diversity of commensal microorganisms that are resident in and on the human body and a number of pathogens are carried by healthy individuals (Prescott et al., 1999). Enteric commensals and pathogens, passed in faeces, are the most obvious forms of biological contamination, and have been most widely studied (McFeters et al., 1993; Delille and Delille, 2000b; Palmgren et al., 2000; Sjoling and Cowan, 2000). Bacteria and fungi resident on human skin and in the mouth, nose and throat are also potential environmental contaminants, but their presence in pristine environments such as Antarctica has yet to be investigated. Bacteria and fungi are shed from the skin continuously as part of normal desquamation (Tannock, 1995) and the microbiota of the mouth and nose are expelled through exhalation, coughing, sneezing and spitting. The distribution of these microorganisms in the environment is potentially more widespread than the dissemination of organisms present in urine and faeces, which are more likely to be concentrated in specific areas. In addition, the new policies of waste containment and removal may greatly reduce the microbiological impact of human wastes in Antarctica.

The most conspicuous effect of bacterial contamination on a pristine environment is the transmission of pathogenic bacteria to endemic species (Palmgren et al., 2000). Viable alien organisms may also disrupt ecosystems at a microscopic level. Microbial communities are the dominant biomass component of Antarctic ecosystems (Vincent, 2000) and invasion of these ecosystems by introduced species could lead to changes in entire community structure. However, the majority of human microbiota are highly adapted to their environment and would be competitively disadvantaged in a foreign environment, especially in the environmental extremes of Antarctica (low temperature and, for some biotypes, low a_w (water activity), and high UV radiation). It has been shown that although relatively high densities of enteric bacteria are found in the seawater surrounding sewage outfalls in Antarctica, titres decrease rapidly with increasing distance from the outfall, suggesting low bacterial viability under these conditions (Delille and Delille, 2000a). The issue of microbial survival (retention of viability) in terrestrial Antarctic environments has been poorly addressed.

3. Persistence of non-indigenous DNA in the environment

Although the Antarctic environment may not favour growth of human commensals, these bacteria may be preserved in a non-viable or dormant state and their DNA may remain intact for long periods, during which they may be inadvertently identified as part of the indigenous community. Smith and McFeters (1993) have shown that enteric bacteria (e.g., *Escherichia coli* and *Salmonella typhimurium)* in untreated sewage released into the Antarctic marine environment can persist in a physiologically active yet non-culturable state. Statham and McMee-kin (1994) demonstrated that faecal bacteria exposed to natural sunlight, during the austral summers in Antarctica, are inactivated and hence would not be detected by plate counts, but their DNA may still be intact. Both bacterial and eukaryotic DNA has been PCR-amplified from several Antarctic sea-ice samples (Brown and Bowman, 2001). Under certain conditions intact DNA can be preserved in the environment for extremely long periods. Reports of DNA being amplified from ancient samples, such as museum specimens (Higuchi et al., 1984; Cano and Poinar, 1993); ancient Egyptian mummies (Ubaldi et al., 1998) and amber (Cano et al., 1993, 1994; Greenblatt et al., 1999), indicates that the ancient DNA was well preserved and protected. Little is known of the stability of exogenous nucleic acids under the conditions found in Antarctic biotopes. The habitually low temperatures and/or low a_w of terrestrial lakes, gravels and ice biotopes argues for high DNA stability and retention. However, UV radiation damages DNA (eg. by formation of thymine dimers) which would render it undetectable by PCR (Upton et al., 1997).

4. Endemic vs. cosmopolitan

A knowledge of whether pristine habitats have been contaminated with non-indigenous species is important for a number of reasons. Firstly, Antarctica and other extreme and pristine habitats harbour largely untapped genetic resources, and are likely to contain material of biotechnological importance (Cowan, 2000). Contamination of these sites with non-indigenous DNA might impede or confuse efforts to harness novel indigenous biomolecules. Secondly, the direct effect of alien species, and the movement of transmissible elements and plasmids from alien to indigenous organisms, may have a deleterious effect on endemic wildlife and communities. Thirdly, for the purposes of monitoring our impact on pristine environments, a regular measure of non-indigenous biological contamination is needed.

It is therefore of utmost importance that the nature of indigenous species is accurately defined. Microbial biogeography is a poorly studied area. A small number of studies in the past decade have investigated whether free-living bacteria are cosmopolitan or endemic (Cho and Tiedje, 2000). Some studies have supported the concept that microorganisms have endemic distributions as for higher organisms (Cas-tenholz, 1996; Fulthorpe et al., 1998; Cho and Tiedje, 2000). In contrast, a high degree (>70%) of DNA-DNA hybridisation has been demonstrated between isolates from thermophilic sites in Alaska and Europe (Stetter et al., 1993) and some genera are found both in the Arctic and Antarctic sea-ice (Staley and Gosink, 1999) supporting the hypothesis that microorganisms are cosmopolitan.

Most Antarctic environments receive microbial propagules from outside the continental boundaries. Vincent (2000) extensively reviews the evolutionary origins of Antarctic microbiota

and discusses pathways of invasion. Natural transport processes that aid the ongoing exchange of microorganisms between Antarctica and the rest of the world include ocean currents, atmospheric circulation, birds, fish and marine mammals. Microorganisms can travel over large distances by such processes. Spore traps in Antarctica, for example, have shown the intermittent arrival of tree pollen from South America (Vincent, 2000). Many organisms found in Antarctica are not uniquely adapted for this ecosystem but originate elsewhere and opportunistically colonise this habitat (Gordon et al., 2000).

The physiology of an isolated microorganism may also provide clues to whether a species is indigenous in an evolutionary sense, or an opportunistic coloniser. Obligate psychrophiles (as opposed to psychrotoler-ants) are more likely to belong to the former category.

5. Indicators of human-derived bacterial contamination

Although it is impossible to ultimately distinguish which organisms are indigenous from those that are introduced, some indication of whether a site has been contaminated by human activities can be gleaned by searching for those organisms normally associated with man.

6. Enteric bacteria

Enteric bacteria are the most studied indicators of human contamination. Sjoling and Cowan (2000) used *E. coli* and *Clostridium clostridiiforme* as indicators of human faecal contamination in Antarctica. They identified low levels of *E. coli* at several occupied locations, but *Clostridium* was only detected in a sewage outfall sample. Other potential indicator species include *Enterococcus* spp., *Peptostreptococcus* (Pre-scott et al., 1999) and *Salmonella* (Cocolin et al., 1998; Palmgren et al., 2000). Certain strains of these species may be specific to humans, whilst others may be found in the normal gut flora of other mammalian and avian hosts (Wang et al., 1996). However, Kreader (1995) demonstrated that PCR-hybridisation assays could distinguish between *Bacteroides distasonis, B. vulga-tus* and *B. thetaiotaomicron* derived from human and non-human faeces in most cases.

7. Normal microbiota of the skin

Malassezia is a basidiomycetous lipophilic yeast commonly isolated from the skin of warm blooded vertebrates (Guillot et al., 2000).There are eight known species of *Malassezia* (Guillot and Gueho, 1995), five of which are common inhabitants of healthy human skin (Schmidt, 1997; Aspiroz et al., 1999; Sugita et al., 2001). These and other *Malassezia* species are also associated with non-human mammals (Aizawa et al., 2001) and one species, *M. pachyder-matis* has been found on the skin of marine mammals (Nakagaki et al., 2000) suggesting that it could be spread between continents without the assistance of man. Other common skin microorganisms include *Staphylococcus* spp., such as *S. epidermis;* Streptococci and *Candida* spp. (Prescott et al., 1999).

8. Buccal and nasal microbiota

There is a huge diversity of microorganisms inhabiting the mouth, throat and nose. Many of these microbes are ejected from the nasal passages, throat and oral cavity during sneezing, coughing or loud talking (Tannock, 1995). Bacteria commonly associated with the nasal cavity include *Staphylococcus aureus,* which inhabits the epithelium lining, (Kloos et al., 1992); *Neisseria* species, which are naturally found in the nasopharynx and include *N. sicca, N. mucosa* and *N. subflava* (Morse and Knapp, 1992; Smith et al., 1999); and Streptococci (e.g., *S. pneumo-niae)* which is dominant in the oropharynx (Hardie and Whiley, 1992; Tannock, 1995). The oral cavity contains different oral surfaces and microhabitats which select for microbes of appropriate attributes (Tannock, 1995). One of the predominant bacteria that inhabits the tongue is *S. salivarius* (Hardie and Whiley, 1992). This Gram-positive bacterium was shown to produce a dextranase enzyme that retards plaque deposition on tooth surfaces (Ohnishi et al., 1995). The mutans group of Streptococci, for example *S. mutans* (Tannock, 1995), inhabit tooth surfaces. Other microbes also naturally found in the oral cavity include *Niesseria* spp., *Corynebacterium, Veillonella, Peptostreptococcus, Lactobacillus*, *Haemophilus* spp. and *Helicobacter* (Gendron et al., 2000).

9. Identification of non-indigenous bacteria in the environment

The majority of studies of non-indigenous bacteria in Antarctica have employed culturing techniques (Miza, 1975; Bruni et al., 1997; Upton et al., 1997; Delille and Delille, 2000a). These methods only identify those viable species that are easily cultured in vitro, and typically fail to isolate organisms that grow poorly in culture or have fastidious growth requirements, such as *Malassezia* spp. (Guillot et al., 2000).It should be noted that currently over 99% of species are believed to be "unculturable" using current technologies (Ward, 1998). Due to the limitations of culturing technologies, the polymerase chain reaction is becoming the preferred method for clinical identification of microorganisms (Wilson et al., 1991; Wang et al., 1996). PCR allows amplification ofviable, non-viable and dormant cells, and primers can be tailored for amplification of specific taxonomic groups.

10. Direct PCR detection

PCR primers can be designed that are homologous to specific genes or oligonucleotides of specific species or strains (Baker et al., submitted forpublication). A number of PCR primers that are designed to amplify specific human microbiota are described in the literature. A selection of these primers is summarised in Table 1. Direct PCR detection uses the PCR reaction as a powerful and sensitive diagnostic tool. In direct detection methods, a negative result infers that there is no DNA homologous to the primer in the sample. However, false negative results can occur due to inappropriate PCR conditions or contamination of the sample by inhibitory substances, such as humic acid (Chandler et al., 1997). Simultaneous amplification of the sample with universal primers, such as those coding for the 16S rRNA gene, or nested PCR using universal primers, serves as a positive control for the amplifiability of the DNA sample. False positive PCR bands and false negative results can also result from poor primer specificity. As little as 70% primer-to-template homology is needed for annealing and elongation (Stern and Holland,

1993). To ensure amplification of DNA homologous to the primer, annealing temperatures must be kept as nearto the melting temperature of the primer as possible (McPherson et al., 1994). In addition, all primers need to be checked against a range of positive and negative controls to confirm specific amplification of the desired template and insure against non-specific amplification of other templates.

Table 1
A selection of taxa-specific primers for the identification of microbial flora associated with the healthy human body

Taxa amplified	Gene amplified	Primer sequence	Size (bp)	References
Skin				
Malassezia spp.	LSU rRNA	5'-GCGCGAAGGTGTCCGAAG-3' 5'-AGCGGAGGAAAAGAAACT-3'	541-579	Guillot et al. (2000)
Gastro-intestinal tract and urinary tract				
Enterococcus spp.	EF-Tu	5'-TACTGACAAACCATTCATGATG-3' 5'-AACTTCGTCACCAACGCGAAC-3'	112	Ke et al. (1999)
E. coli	16s rRNA	5'-GGGAGTAAAGTTAATACCTTTGCTC-3' 5 -TTCCCGAAGGCACATTCT-3	584	Tsen et al. (1998)
E. coli	16s rRNA	5 -GACCTCGGTTTAGTTCACAGA-3 5 -CACACGCTGACGCTGACCA-3	585	Wang et al. (1996)
C. clostridiiforme	16s rRNA	5 -CCGCATGGCAGTGTGTGAAA-3 5 -CTGCTGATAGAGCTTTACATA-3	255	Wang et al. (1996)
Peptostreptococcus productus	16s rRNA	5 -AACTCCGGTGGTATCAGATG-3 5 -GGGGCTTCTGAGTCAGGTA	268	Wang et al. (1996)
Salmonella	invA	5 -GCTGCGCGCGAACGGCGAAG-3 5 -TCCCGGCAGAGTTCCCATT-3	389	Cocolin et al. (1998)
Nose and mouth				
S. salivarius	dextranase	5 -CAGTGTGGCTAAAGACACTATT-3 5 -TGTCCGCATAACCCTCTTCATT-3		This paper
H. pylori	genomic	5"-CCCTCACGCCATCAGTCCCAAAAA-3' 5 -AAGAAGTCAAAAACGCCCCAAAAC-3	860	Song et al. (2000)
S. pnemoniae	pnemolysin	5 -AGCGATAGCTTTCTCCAAGTGG-3 5 -CTTAGCCAACAAATCGTTTACCG-3	74	Greiner et al. (2001)
S. mutans	gtfB	5 -AAAGCAGATTCTAATGAATCGA-3 5 -AATGTAAAATTTTGCCATCAGC-3	517	Oho et al. (2000)
S. aureus	ent B	5 -GAGAGTCAACCAGATCCTAAACCAG-3 5 -ATACCAAAAGCTATTCTCATTTTCT-3	593	Wilson et al. (1991)
Neisseria	16S rRNA	5 -GTCATGAAGCATACCGTGGT-3 5'-CATAAGAGTTTGATCCTGGCT-3'	1355	Smith et al. (1999)

Primer design should be based on target specific homologies, inferred from sequence data, but it is important to note that the sequence data available in databases is not reflective of natural biodiversity. Primers can only be designed on the basis of available sequence data and thus although primers may be designed to be specific to DNA in the database they may also anneal to the DNA of other unsequenced organisms. It is thus very important that all potential primers are tested with controls in the laboratory prior to their use as diagnostic tools.

11. Other PCR-based detection methods

An alternative strategy to direct detection is to use PCR as a preliminary step, to amplify a gene or other DNA fragment, then to use supplementary methods to identify the organism more specifically. These methods are obviously more time consuming and costly than direct methods, but potentially provide less ambiguous results. In addition, less specific PCR primers can be used that amplify families or genera rather than specific species or strains.

12. Partial sequencing

Partial sequencing of PCR products can be highly informative if the DNA region chosen is suitably variable. Highly conserved genes such as those coding for 16S rRNA are ideal for identifying organisms from different genera, but the level of base conservation in some parts of the gene will be too great to resolve species differences. Goto et al. (2000) effectively used partial sequence data from hypervariable regions 1 and 2 of 16S rDNA to identify a number of *Bacillus* species. Similarly, Monstein et al. (1998) has used partial sequencing of variable regions V4 and V9 of 16S rDNA to identify *Enterococcus* species.

13. Restriction enzyme analysis

The use of restriction enzyme analysis methodologies, such as ARDRA and RFLP, has also been effective to identify human commensal species (Guil-lotetal.,2000; Guptaetal.,2000).After PCR, amplicons are digested with one or more restriction enzymes to form a digest fragment pattern. If the restriction enzymes are chosen carefully, restriction patterns may be species or strain specific. False negative bands can, however, result from inadequate digestion leading to the formation of different banding patterns for identical samples. Furthermore, if restriction enzymes are poorly chosen, different species may have identical restriction patterns.

Other post-PCR methods for identification include DGGE (Millar et al., 1996; Watanabe et al., 2001); tRNA-ILPs (Welsh and McClelland, 1992; Maes et al., 1997) and Southern Hybridisation (Feder et al., 2001).

14. Conclusions

PCR is a powerful diagnostic tool that has been used effectively in the identification of human commensal microbiota in the environment (Kreader, 1995; Upton et al., 1997; Schvoerer et al., 2000; Sjoling and Cowan, 2000; Feder et al., 2001) andinclinical samples (Makimura et al.,1994; Wang et al., 1996; Monstein et al., 1998; Ke et al., 1999; Guillot et al., 2000; Oho et al., 2000; Prasad et al., 2000; Song et al., 2000; Greiner et al., 2001; Sugita et al., 2001). The successful application of PCR for identification of non-indigenous organisms from the pristine habitats of Antarctica is less certain. The two major " problems" are: (i) non-indigenous microorganisms may be transported to and distributed in the Antarctic continent by natural processes, and (ii) specific

phylotypes may not be detectable due to their low concentrations and heterogeneous distribution. The former may be resolved by the appropriate inclusion of 'pristine' samples in studies of the occurrence and distribution of known non-indigenous phylotypes. In addition, the designation of novel isolates as ' indigenous' or 'non-indigenous' may be reasonably inferred from the physiological characteristics of the isolate, where evidence of psychrophilic or psychrotolerant growth characteristics implies adaptation to low temperature (i.e., Antarctic) environmental conditions. The latter may be addressed by the extent and magnitude of the in situ sampling program, and by the development and/ or use of very high sensitivity PCR protocols.

Where PCR is to be used reliably for detection of non-indigenous organisms in pristine habitats such as the Antarctic Dry Valleys, the following three criteria (at least) should be met: (i) great care must be exercised in the choice of appropriate primers, (ii) extensive laboratory trials to establish their specificity should be carried out, and (iii) stringently selected positive and negative controls must be included in the study.

Table 1 provides a list of published species-specific primers, designed for analytical use. Homology testing using BLAST indicates that these primers have lower homology scores to other templates than to the species and genera listed (with the exception of the reverse *Malassezia* primer), suggesting that they are indeed appropriate for species-specific analytical applications. However, we believe that it is critical that specificity is confirmed experimentally. As a minimum requirement, we suggest that prior to use in analysis of environmental samples, primers should be tested against type-cultures of the target strains and species (positive controls), including 'seeded' environmental samples, and against extracts of pristine samples (negative controls).

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References

Aizawa, T., Kano, R., Nakamura, Y., Watanabe, S., Hasegawa, A., 2001. The genetic diversity of clinical isolates of*Malassezia pa-chydermatis* from dogs and cats. Med. Mycol. 39 (4), 329 -334.

Aspiroz, C., Moreno, L.A., Rezusta, A., Rubio, C., 1999. Differentiation of three biotypes of *Malassezia* species on human normal skin. Correspondence with *M globosa, M. sympodialis* and *M. restricta.* Mycopathologica 145 (2), 69 -74.

Baker, G.C., Whiting, S.J., Cowan, D.A., submitted for publication. Review and re-analysis of Domain-specific 16S primers. FEMS Microbiol. Ecol.

Brown, M.V., Bowman, J.P., 2001. A molecular phylogenetic survey of sea-ice microbial communities. FEMS Microbiol. Ecol.35, 267-275.

Bruni, V., Maugeri, T.L., Monticelli, L., 1997. Faecal pollution indicators in Terra Nova Bay (Ross Sea, Antarctica). Mar. Pollut. Bull. 34, 908 -912.

Cano, R.J., Poinar, H.N., 1993. Rapid isolation of DNA from fossil and museum specimens suitable for PCR. Biotechniques 15 (3), 432 -434.

Cano, R.J., Poinar, H.N., Pieniazek, N.J., Acra, A., Poinar Jr., G.O. 1993. Amplification and sequencing of DNA from a 120 -135-million-year-old weevil. Nature 363 (6429), 536-538.

Cano, R.J., Borucki, M.K., Higby-Schweitzer, M., Poinar, H.N., Poinar Jr., G.O., Pollard, K.J. 1994. *Bacillus* DNA in fossil bees: an ancient symbiosis? Appl. Environ. Microbiol. 60 (6), 2164-2167.

Castenholz, R.W., 1996. Endemism and biodiversity of thermo-philic cyanobacteria. Nova Hedwig. Beih. 112, 33 -47.

Chandler, D.P., Schreckhise, R.W., Smith, J.L., Bolton Jr., H. 1997. Electroelution to remove humic compounds from soil DNA and RNA extracts. J. Microbiol. Methods 28, 11 -19.

Cho, J.-C., Tiedje, J.M., 2000. Biogeography and degree of ende-micity of fluorescent *Pseudomonas* strains in soil. Appl. Environ. Microbiol. 66 (12), 5448 -5456.

Cocolin, L., Manzano, M., Cantoni, C., Comi, G., 1998. Use of polymerase chain reaction and restriction enzyme analysis to directly detect and identify *Salmonella typhimurium* in food. J. Appl. Microbiol. 85, 673-677.

Cowan, D.A., 2000. Microbial genomes—the untapped resource. TIBTECH 18, 14-16.

Delille, D., 2000. Response of Antarctic soil bacterial assemblages to contamination by diesel fuel and crude oil. Microbiol. Ecol. 40 (2), 159-168.

Delille, D., Delille, E., 2000a. Distribution of enteric bacteria in Antarctic seawater surrounding Dumont d'Urville permanent station (Adelie land). Mar. Pollut. Bull. 40 (10), 869-872.

Delille, D., Delille, B., 2000b. Field observations on the variability of crude oil impact on the indigenous hydrocarbon-degrading bacteria from sub-Antartic intertidal sediments. Mar. Environ. Res. 49 (5), 403-417.

Enzenbacher, D.J., 1992. Antarctic tourism and environmental concerns. Mar. Pollut. Bull. 25 (9-12), 258-265.

Feder,I.,Nietfeld,J.C.,Galland,J.,Yeary,T.,Sargeant,J.M., Oberst, R., Tamplin, M.L., Luchansky, J.B., 2001. Comparison of cultivation and PCR hybridization for detection of *Salmonella* in porcine fecal and water samples. J. Clin. Microbiol. 39 (7),2477-2484.

Fulthorpe, R.R., Rhodes, A.N., Tiedje, J.M., 1998. High levels of endemicity of 3-chlorobenzoate-degrading soil bacteria. Appl. Environ. Microbiol. 64, 1620-1627.

Gendron, R., Grenier, D., Maheu-Robert, L.F., 2000. The oral cavity as a reservoir of bacterial pathogens for focal infections.Microbes Infect. 2 (8), 897-906.

Gordon, D.A., Priscu, J., Giovannoni, S., 2000. Origin and phylog-eny of microbes living in permanent Antarctic lake ice. Microbiol. Ecol. 39 (3), 197-202.

Goto, K., Omura, T., Hara, Y., Sadaie, Y., 2000. Application ofthe partial 16S rDNA sequence as an index for rapid identificaton of species in the genus *Bacillus*. J. Gen. Appl. Microbiol. 46,1-8.

Greenblatt, C.L., Davis, A., Clement, B.G., Kitts, C.L., Cox, T.,Cano, R.J., 1999. Diversity of microorganisms isolated from amber. Microbiol. Ecol. 38 (1), 58-68.

Greiner, O., Day, P.J., Bosshard, P.P., Imeri, F., Altwegg, M., Nadal, D., 2001. Quantitative Detection of *Streptococcus pnemoniae* in nasopharyngeal secretions by real-time PCR. J. Clin. Microbiol. 39 (9), 3129-3134.

Guillot, J., Gueho, E., 1995. The diversity of *Malassezia* yeasts confirmed by ribosomal-RNA sequence and nuclear-DNA comparisons. Antonie van Leeuwenhoek Int. J. Gen. M. 67 (3), 297-314.

Guillot, J., Deville, M., Berthelemy, M., Provost, F., Gueho, E., 2000. A single PCR restriction endonuclease analysis for rapid identification of *Malassezia* species. Lett. Appl. Microbiol. 31 (5), 400-403.

Gupta, A.K., Kohli, Y., Summerbell, R.C., 2000. Molecular differentiation of seven *Malassezia* species. Clin. Microbiol. 38 (5), 1869 -1875.

Hardie, J.M., Whiley, R.A., 1992. The genus *Streptococcus*-Oral. In: Balows, A., Triiper, H.G., Dworkin, M., Harder, W., Schlei-fer, K. (Eds.), The Prokaryotes, vol. 2. Springer-Verlag, New York, pp. 1421-1449.

Higuchi, R., Bowman, B., Freiberger, M., Ryder, O.A., Wilson, A.C., 1984. DNA sequences from the quagga, an extinct member of the horse family. Nature 312 (5991), 282-284.

Ke, D., Picard, F.J., Martineau, F., Menard, C., Roy, P.H., Ouelette, M.G., Bergeron, M.G., 1999. Development of a PCR assay for rapid detection of enterococci. J. Clin. Microbiol. 37 (11), 3497-3503.

Kloos, W.E., Schleifer, K., Gotz, F., 1992. The genus *Staphylococcus.* In: Balows, A., Truper, H.G., Dworkin, M., Harder, W., Schleifer, K. (Eds.), The Prokaryotes, vol. 2. Springer-Verlag, New York, pp. 1369-1420.

Kreader, C.A., 1995. Design and evaluation of *Bacteroides* DNA probes for the specific detection of human fecal pollution. Appl. Environ. Microbiol. 61 (4), 1171-1179.

Maes, N., De Gheldre, Y., De Ryck, R., Vaneechoutte, M., Meug-nier, H., Ettienne, J., Struelens, M.J., 1997. Rapid and accurate identification of Staphylococcus species by tRNA intergenic spacer length polymorphism analysis. J. Clin. Microbiol. 35 (10), 2477-2481.

Makimura, K., Murayama, S.Y., Yamaguchi, H., 1994. Detection of a wide-range of medically important fungi by the polymerase chain-reaction. J. Med. Microbiol. 40 (5), 358-364.

McFeters, G.A., Barry, J.P., Howington, J.P., 1993. Distribution of enteric bacteria in Antarctic seawater surrounding a sewage outfall. Water Res. 27, 645 -650.

McPherson, M.J., Quirk, P., Taylor, G.R., 1994. PCR I: A Practical Approach Oxford university Press, Oxford, England.

Millar, M.R., Linton, C.J., Cade, A., Glancy, D., Hall, M., Jalal, H.,1996. Application of 16S rRNA gene PCR to study bowel flora of preterm infants with and without necrotizing enterocolitis. J. Clin. Microbiol. 34 (10), 2506-2510. Miza, T., 1975. Clostridia in the soil ofAntarctica. Jpn. J. Med. Sci. Biol. 28, 201 -213.

Monstein, H.J., Quednau, M., Samuelson, A., Ahrne, S., Isaksson, B., Jonasson, J., 1998. Division of genus *Enterococcus* into species groups using PCR-based molecular typing methods. Microbiology 144, 1171-1179.

Morse, S.A., Knapp, J.S., 1992. The genus *Neisseria.* In: Balows, A., Triiper, H.G., Dworkin, M., Harder, W., Schleifer, K. (Eds.), The Prokaryotes, vol. 3. Springer-Verlag, New York, pp. 2495-2529.

Nakagaki, K., Hata, K., Iwata, E., Takeo, K., 2000. *Malassezia pachydermatis* isolated from South America sea lion *(Otariabyronia)* with dermatitis. J. Vet. Med. Sci. 62 (8), 901 -903. Ohnishi, Y., Kubo, S., Ono, Y., Nozaki, M., Gonda, Y., Okano, H.,

Matsuya, T., Matsushiro, A., Morita, T., 1995. Cloning and sequencing of the gene coding for dextranase from *Streptococcus salivarius.* Gene 156, 93 -96.

Oho, T., Yamashita, Y., Shimazaki, Y., Kushiyama, M., Koga, T., 2000. Simple and rapid detection of *Streptococcus mutans* and *Streptococcus sobrinus* in human saliva by polymerase chain reaction. Oral Microbiol. Immunol. 15 (4), 258-262.

Palmgren, H., McCafferty, D., Aspan, A., Broman, T., Sellin, M., Wollin, R., Bergstrom, S., Olsen, B., 2000. *Salmonella* in sub-Antarctica: low heterogeneity in *Salmonella* serotypes in South Georgian seals and birds. Epidemiol. Infect. 125 (2), 257-262.

Prasad, K.N., Kumar, R., Tiwari, D.P., Mishra, K.K., Ayyagari, A., 2000. Comparison of various conventional methods with a pol-ymerase chain reaction assay for detecting methicillin-resistant and susceptible *Staphylococcus aureus* strains. Indian J. Med. Res. 112, 198-202.

Prescott, L.M., Harley, J.P., Klein, D.A., 1999. Microbiology, 4th ed. WCB McGraw-Hill, Boston.

Schmidt, A., 1997. *Malassezia furfur:* a fungus belonging to the physiological skin flora and its relevance in skin disorders. Cutis 59 (1), 21-24.

Schvoerer, E., Bonnet, F., Dubois, V., Cazaux, G., Serceau, R., Fleury, H.J.A., Lafon, M.E., 2000. PCR detection of human enteric viruses in bathing areas, waste waters and human stools in southwestern France. Res. Microbiol. 151 (8), 693-701.

Sjoling, S., Cowan, D.A., 2000. Detecting human bacterial contamination in Antarctic soils. Polar Biol. 23 (9), 644 -650.

Smith, J.J., McFeters, G.A., 1993. Survival and recoverability of enteric bacteria exposed to the antarctic marine environment. Antarct. J. 28, 120-123.

Smith, N.H., Holmes, E.C., Donovan, G.M., Carpenter, G.A., Spratt, B.G., 1999. Networks and groups within the genus *Neis-seria:* analysis of *argF, recA, rho,* and 16S rRNA sequences from human *Neisseria* species. Mol. Biol. Evol. 16 (6), 773 -783.

Song, Q., Lange, T., Spahr, A., Adler, G., Bode, G., 2000. Characteristic distribution pattern of *Helicobacter pylori* in dental plaque and saliva detected by nested PCR. J. Med. Microbiol. 49 (4), 349-353.

Staley, J.T., Gosink, J.J., 1999. Poles apart: biodiversity and bio-geography. Annu. Rev. Microbiol. 53, 189 -215.

Statham, J.A., McMeekin, T.A., 1994. Survival of faecal bacteria in Antarctic coastal waters. Antarct. Sci. 6 (3), 333 -338.

Stern, C.D., Holland, P.W., 1993. Essential Developmental Biology: A Practical Approach Oxford Univ. Press, Oxford, England.

Stetter, K.O., Hubert, R., Blochl, E., Kurr, M., Eden, R.D., 1993. *Hyperthermophilic archaea* are thriving in deep North sea and Alaskan oil reservoirs. Nature 365, 743 -745.

Sugita, T., Suto, H., Unno, T., Tsuboi, R., Ogawa, H., Shinoda, T., Nishikawa, A., 2001. Molecular analysis of *Malassezia* micro-flora on the skin of atopic dermatitis patients and healthy subjects. J. Clin. Microbiol. 39 (10), 3486-3490.

Tannock, G.W., 1995. Normal Microflora: An Introduction to Microbes Inhabiting the Human Body Chapman & Hall, London.

Tsen, H.Y., Lin, C.K., Chi, W.R., 1998. Development and use of 16S rRNA gene targeted PCR primers for identification of *E. coli* in water. J. Appl. Microbiol. 85, 554-560.

Ubaldi, M., Luciani, S., Marota, I., Fornaciari, G., Cano, R.J., Rollo, F., 1998. Sequence analysis of bacterial DNA in the colon of an Andean mummy. Am. J. Phys. Anthropol. 107 (3), 285 -295.

Upton, M., Pennington, T.H., Haston, W., Forbes, K.J., 1997. Detection of human commensals in the area around an Antarctic research station. Antarct. Sci. 9 (2), 156 -161.

Vincent, W.F., 2000. Evolutionary origins of Antarctic micro-biota: invasion, selection and endemism. Antarct. Sci. 12 (3),374-385.

Wang, R.-F., Cao, W.-W., Cerniglia, C.E., 1996. PCR detection and quantification of predominant anerobic bacteria in human and animal fecal samples. Appl. Environ. Microbiol. 62 (4), 1242-1247.

Ward, D.M., 1998. A natural species concept for prokaryotes. Curr.Opin. Microbiol. 1 (3), 271 -277.

Watanabe, K., Kodama, Y., Harayama, S., 2001. Design and evaluation of PCR primers to amplify bacterial 16S ribosomal DNA fragments used for community fingerprinting. J. Microbiol. Methods 44, 253-262.

Welsh, J., McClelland, M., 1992. PCR-amplified length polymorphisms in tRNA intergenic spacers for categorizing staphylococci. Mol. Microbiol. 6 (12), 1673 -1680.

Wilson, I.G., Cooper, J.E., Gilmour, A., 1991. Detection of enter-otoxigenic *Staphylococcus aureus* in dried skimmed milk: use of the polymerase chain reaction for amplification and detection of Staphylococcal enterotoxin genes *entB* and *entC1* and the Thermonuclease gene *nuc*. Appl. Environ. Microbiol. 57 (6), 1793-1798.