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ORIGINAL ARTICLE

# Correct species identification (reclassification in CNCTC) of strains of *Staphylococcus intermedius*-group can improve an insight into their evolutionary history

Lucia Mališová [1] · Renáta Šafránková [2,3] · Jana Kekláková [4] · Petr Petráš [4] · Helena Žemličková [1,3] · Vladislav Jakubů [1,3]

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## Abstract

A group of 59 putative strains of *Staphylococcus intermedius*/*Staphylococcus pseudintermedius* deposited in the Czech National Collection of Type Cultures (CNCTC, National Institute for Public Health, Prague, Czech Republic) and the National Reference Laboratory for Staphylococci (NRL for Staphylococci, National Institute for Public Health, Prague, Czech Republic) was reclassified using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). There the biggest human collection of *S. pseudintermedius* in Europe was analysed; 44 samples (75%) were of human origin. Twenty-two percent ($n = 13$) of the strains were isolated from animals, and two staphylococci were of unknown origin. This study revealed the prevalence of *Staphylococcus pseudintermedius* (94%, $n = 53$) vs. *Staphylococcus intermedius* (6%, $n = 6$) in the collection of human and veterinary staphylococci after reclassification. Results of PCR-RFLP analysis were verified by comparison with a repetitive element sequence-based polymerase chain reaction (Rep-PCR) analysis on 26 (44%) randomly selected strains. Due to a low-resolution ability of PCR-RFLP to separate *Staphylococcus intermedius* from *Staphylococcus delphini*, **four** isolates of *Staphylococcus intermedius* were biochemically verified further to exclude the presence of *Staphylococcus delphini* in the collection. Our results indicate that *S. intermedius* and *S. pseudintermedius* have occurred independently over an age-long period of their co-evolution.

## Introduction

Staphylococci routinely colonising humans and animals can cause a wide range of different purulent and toxin-mediated diseases (Murugaiyan et al. 2014). Due to a high degree of their phenotypic similarity, it is difficult to differentiate some of them into species. *Staphylococcus intermedius* was for the first time described by Hájek as a new staphylococcal species isolated from pigeons, dogs, minks, and horses in 1976 (Hájek 1976). Already, Hájek had pointed at certain phenotypical variability in the group of this pathogen, what was later confirmed by describing other species similar to each other. Except for *S. intermedius* (Bannoehr et al. 2007; Devriese et al. 2009), there is also *Staphylococcus pseudintermedius* and *Staphylococcus delphini* belonging to the, so called, *Staphylococcus intermedius*-group (SIG) (Murugaiyan et al. 2014). Members of the SIG are zoonotic pathogens mainly a part of skin microbiota of various organisms able to cause a variety of animal infections (Sasaki et al. 2007), and due to the transmission by pets, they can also cause human infections (Zubeir et al. 2007; Bannoehr et al. 2009; Murugaiyan et al. 2014). Owing to a proved transmission of *S. pseudintermedius* from animals (dogs and cats) to humans (veterinary staff, animal owners) (Zubeir et al. 2007; Bond and Loeffler 2012), a special kind of attention should be given to this group not only in veterinary but

✉ Lucia Mališová
lucia.malisova@szu.cz

[1] National Reference Laboratory for Antibiotics, National Institute of Public Health, Prague, Czech Republic

[2] Czech National Collection of Type Cultures, National Institute of Public Health, Prague, Czech Republic

[3] Faculty of Medicine in Hradec Králové, Charles University, Hradec Králové, Czech Republic

[4] National Reference Laboratory for Staphylococci, National Institute of Public Health, Prague, Czech Republic

also in human medicine (Chrobak et al. 2011; Kmieciak and Szewczyk 2018).

Based on the results of biochemical testing, DNA G+C contents or DNA–DNA hybridization, *S. pseudintermedius* (isolated from animals) was described as a separate strain in 2005 by Devries (Devriese et al. 2005). On the other hand, *S. delphini* was isolated from dolphins much earlier, in the year 1988 (Varaldo et al. 1988). Members of the SIG are characterised by a similar large colonies without pigmentation and growth under aerobic conditions. SIG strains usually produce β-hemolysin, catalase, alkaline phosphatase, urease, and acid from sugars (mannose, sucrose) and are able to reduce nitrate (Jorgensen et al. 2015). Based on mentioned facts, it can be supposed that a classical biochemical differentiation may result in insufficient species identification (Murugaiyan et al. 2014). An absence of commercial kits able to distinguish members of this group (Sasaki et al. 2007) can also cause that differentiation of the SIG members into species is a difficult task. Moreover, due to a sharing of a high level of nucleotide identity at the 16S ribosomal DNA (rDNA) gene in this family (Slettemeås et al. 2010), sequencing of this gene fails too (Devriese et al. 2009).

The importance of the SIG members' differentiation lies in probably higher pathogenicity of *S. pseudintermedius* compared to *S. intermedius*. Comparative analysis of the whole genomes of the SIG identified variation in the content of mobile genetic elements, cell wall-associated proteins, and iron and sugar transporters, and especially, *S. pseudintermedius* contained more genetic transposons involved in multidrug resistance than other members of the SIG (Ben Zakour et al. 2012). In addition, there is still an increasing number of studies confirming that *S. pseudintermedius* is a human pathogen (Van Hoovels et al. 2006; Viau et al. 2015) associated with methicillin (Fitzgerald 2009; Stegmann et al. 2010; Starlander et al. 2014), penicillin, tetracycline, and macrolide resistance (Ruzauskas et al. 2016; Ventrella et al. 2017).

Coagulase-positive staphylococci can be distinguished using different methods, i.e. multiplex PCR, sequencing of *nuc* gene (Sasaki et al. 2010), and a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. PCR-RFLP is a cheap, fast, and easy-way technique based on phosphoacetyltransferase (*pta*) gene amplification followed by a digestion with a specific endonuclease (Bannoehr et al. 2009). The most of indefinitely identified *S. intermedius*/*S. pseudintermedius* were deposited in the CNCTC and NRL for Staphylococci. Therefore, the aims of this study were to verify the effectiveness of PCR-RFLP in differentiation of the SIG, to reclassify all strains belonging to this family, and to look a little into their history.

## Method

### Bacterial strains

Altogether, 59 strains previously identified as *S. intermedius* or as a couple *S. intermedius*/*S. pseudintermedius* (Table 1) deposited at the department of CNCTC and NRL for Staphylococci were identified with standard phenotypic methods that include production of tube coagulase, heat-stable nuclease, acetoin, pyrrolidonyl arylamidase, β-galactosidase, α- and β-hemolysins, and expression of clumping factor. Strains were isolated preferentially from humans ($n = 44$) and animals ($n = 13$): pigeon, dogs, cat, and pine marten.

### Genomic-DNA isolation and PCR-RFLP analysis

All isolates were grown on nutrient agar (NA; Oxoid) under aerobic condition for 24 h at 36 °C. The list of all samples is in Table 1. Genomic-DNA extraction of all isolates was carried out with a bacterial genomic-DNA purification kit (Sigma-Aldrich) according to the manufacturer's instructions. DNA amplification of a 320-bp fragment of *pta* gene was performed with 25-μL volume containing 0.2-nmol/L concentration of oligonucleotide primers (F: 5′–AAA GAC AAA CTT TCA GGT AA–3′ and R: 5′–GCA TAA ACA AGC ATT GTA CCG–3′) with Taq DNA polymerase reaction master mix (BioLabs). The reaction mixture was subjected to initial denaturation 95 °C for 2 min followed by 30 cycles of 95 °C for 1 min, 53 °C for 1 min, and 72 °C for 1 min, with a final incubation of 72 °C for 7 min. PCR amplicons were incubated with 5 U of *Mbo* I enzyme (Thermo Scientific) enriched by 2.5 μL of 10× digestion buffer for 2 h; the digestion products were consequently resolved in 2% agarose by electrophoresis.

### Biochemical differentiation

After PCR-RFLP analysis, biochemical testing involving DNase, trehalose, and acetoin detection was used for differentiation of four isolates of *S. intermedius* from *S. delphini*. DNase production was tested on a 48-h-old culture on DNase agar (Oxoid) by adding HCl (1 mol/L); DNase positive samples were manifested by the brightening around colonies. Detection of trehalose utilisation was done using conventional tube test with a bromothymol blue. Acetoin production was performed by a Voges-Proskauer test (VP test) according to the manufacturer's instructions (Erba Lachema).

#### Rep-PCR

Rep-PCR was performed as described previously (Švec et al. 2010) at the Department of Experimental Biology, Czech Collection of Microorganisms (Brno, Czech Republic). A

**Table 1** List of strains included in the study. Staphylococci were obtained from the CNCTC and NRL for Staphylococci

| ID of isolate | Year of isolation/ identification | Origin | Strains originally deposed in the CNCTC and NRL for Staphylococci | Species after PCR-RFLP reclassification | Results of Rep-PCR[c] |
|---|---|---|---|---|---|
| 5681[ad] | 1975 | Pigeon | *S. intermedius* | *S. intermedius* | *S. intermedius* |
| 6046[a] | 1979 | unknown | *S. intermedius* | *S. intermedius* | – |
| 7130[a] | 1979 | Pine marten | *S. intermedius* | *S. intermedius* | – |
| 6047[a] | 1985 | Dog | *S. intermedius* | *S. pseudintermedius* | – |
| 6048[a] | 1985 | Dog | *S. intermedius* | *S. pseudintermedius* | – |
| 6049[a] | 1985 | Dog | *S. intermedius* | *S. intermedius* | – |
| 6050[a] | 1985 | Dog | *S. intermedius* | *S. pseudintermedius* | – |
| 6051[a] | 1985 | Dog | *S. intermedius* | *S. pseudintermedius* | – |
| 6052[a] | 1985 | Dog | *S. intermedius* | *S. pseudintermedius* | – |
| 6053[a] | 1986 | Dog | *S. intermedius* | *S. pseudintermedius* | – |
| 6054[a] | 1986 | Dog | *S. intermedius* | *S. pseudintermedius* | – |
| 6718[a] _ 00/523[b] | 2000 | Unknown | *S. intermedius* | *S. pseudintermedius* | – |
| 00/227[b] | 2000 | Human | *S. intermedius* | *S. pseudintermedius* | *S. pseudintermedius* |
| 00/470[b] | 2000 | Human | *S. intermedius* | *S. pseudintermedius* | *S. pseudintermedius* |
| 00/621[b] | 2000 | Human | *S. intermedius* | *S. pseudintermedius* | *S. pseudintermedius* |
| 00/892[b] | 2000 | Human | *S. intermedius* | *S. pseudintermedius* | *S. pseudintermedius* |
| 00/984[b] | 2000 | Human | *S. intermedius* | *S. pseudintermedius* | *S. pseudintermedius* |
| 01/704[b] | 2001 | Human | *S. intermedius* | *S. pseudintermedius* | – |
| 01/719[b] | 2001 | Human | *S. intermedius* | *S. pseudintermedius* | *S. pseudintermedius* |
| 01/777[b] | 2001 | Human | *S. intermedius* | *S. pseudintermedius* | *S. pseudintermedius* |
| 01/824[b] | 2001 | Human | *S. intermedius* | *S. pseudintermedius* | *S. pseudintermedius* |
| 01/900[b] | 2001 | Human | *S. intermedius* | *S. pseudintermedius* | *S. pseudintermedius* |
| 02/013[b] | 2002 | Human | *S. intermedius* | *S. pseudintermedius* | *S. pseudintermedius* |
| 02/139[b] | 2002 | Human | *S. intermedius* | *S. pseudintermedius* | *S. pseudintermedius* |
| 02/172[b] | 2002 | Human | *S. intermedius* | *S. pseudintermedius* | *S. pseudintermedius* |
| 02/179[b] | 2002 | Human | *S. intermedius* | *S. pseudintermedius* | *S. pseudintermedius* |
| 02/218[b] | 2002 | Human | *S. intermedius* | *S. pseudintermedius* | *S. pseudintermedius* |
| 02/264[b] | 2002 | Dog | *S. intermedius* | *S. pseudintermedius* | *S. pseudintermedius* |
| 02/423[b] | 2002 | Human | *S. intermedius* | *S. pseudintermedius* | *S. pseudintermedius* |
| 02/438[b] | 2002 | Human | *S. intermedius* | *S. pseudintermedius* | *S. pseudintermedius* |
| 02/573[b] | 2002 | Dog | *S. intermedius* | *S. pseudintermedius* | – |
| 02/589[b] | 2002 | Human | *S. intermedius* | *S. pseudintermedius* | *S. pseudintermedius* |
| 02/682[b] | 2002 | Human | *S. intermedius* | *S. pseudintermedius* | *S. pseudintermedius* |
| 02/718[b] | 2002 | Human | *S. intermedius* | *S. pseudintermedius* | *S. pseudintermedius* |
| 03/033[b] | 2003 | Human | *S. intermedius* | *S. pseudintermedius* | *S. pseudintermedius* |
| 7634[a] _ 03/087[b] | 2003 | Human | *S. intermedius* | *S. intermedius* | *S. intermedius* |
| 03/215[b] | 2003 | Human | *S. intermedius* | *S. pseudintermedius* | *S. pseudintermedius* |
| 03/378[b] | 2003 | Human | *S. intermedius* | *S. pseudintermedius* | *S. pseudintermedius* |
| 03/420[b] | 2003 | Human | *S. intermedius* | *S. pseudintermedius* | – |
| 04/304[b] | 2004 | Human | *S. intermedius* | *S. pseudintermedius* | – |
| 04/670[b] | 2004 | Human | *S. intermedius* | *S. pseudintermedius* | – |
| 05/085[b] | 2005 | Human | *S. intermedius* | *S. pseudintermedius* | – |
| 05/560[b] | 2005 | Human | *S. intermedius* | *S. pseudintermedius* | – |
| 05/886[b] | 2005 | Human | *S. intermedius*/*S. pseudintermedius* | *S. pseudintermedius* | – |
| 06/478[b] | 2006 | Human | *S. intermedius*/*S. pseudintermedius* | *S. pseudintermedius* | – |
| 06/528[b] | 2006 | Human | *S. intermedius*/*S. pseudintermedius* | *S. pseudintermedius* | – |
| 06/915[b] | 2006 | Human | *S. intermedius*/*S. pseudintermedius* | *S. pseudintermedius* | – |

**Table 1** (continued)

| ID of isolate | Year of isolation/ identification | Origin | Strains originally deposed in the CNCTC and NRL for Staphylococci | Species after PCR-RFLP reclassification | Results of Rep-PCR[c] |
|---|---|---|---|---|---|
| 07/514[b] | 2007 | Human | *S. intermedius*/*S. pseudintermedius* | *S. intermedius* | – |
| 07/849[b] | 2007 | Human | *S. intermedius*/*S. pseudintermedius* | *S. pseudintermedius* | – |
| 07/860[b] | 2007 | Human | *S. intermedius*/*S. pseudintermedius* | *S. pseudintermedius* | – |
| 08/1041[b] | 2008 | Human | *S. intermedius*/*S. pseudintermedius* | *S. pseudintermedius* | – |
| 08/866[b] | 2008 | Human | *S. intermedius*/*S. pseudintermedius* | *S. pseudintermedius* | – |
| 09/627[b] | 2009 | Human | *S. intermedius*/*S. pseudintermedius* | *S. pseudintermedius* | – |
| 09/932[b] | 2009 | Human | *S. intermedius*/*S. pseudintermedius* | *S. pseudintermedius* | – |
| 10/274[b] | 2010 | Human | *S. intermedius*/*S. pseudintermedius* | *S. pseudintermedius* | – |
| 10/857[b] | 2010 | Human | *S. intermedius*/*S. pseudintermedius* | *S. pseudintermedius* | – |
| 11/0038[b] | 2012 | Human | *S. intermedius*/*S. pseudintermedius* | *S. pseudintermedius* | – |
| 12/386[b] | 2012 | Human | *S. intermedius*/*S. pseudintermedius* | *S. pseudintermedius* | – |
| 7446[ad] | 2015 | Cat | *S. pseudintermedius* | *S. pseudintermedius* | *S. pseudintermedius* |

[a] Isolates deposited in the CNCTC

[b] Isolates obtained from the NRL for Staphylococci

[c] Results of Rep-PCR (Švec et al. 2010)

[d] Type strain

fresh DNA was isolated from the SIG. Rep-PCR was performed using the $(GTG)_5$-specific primer at appropriate thermal conditions. After amplification of repetitive sequences, products were separated in 2% agarose gel. Electrophoretic profiles were analysed by Bionumerics v. 6.0 software (Applied Maths) (Švec et al. 2010).

## Results and discussion

The objectives of this article were verification of effectiveness of PCR-RFLP in the differentiation of the SIG and reclassification of this family. PCR-RFLP is a molecular typing method that can be used in mapping studies of bacteria and humans. It enables us to analyse a very complex mixture of DNA with discriminatory power comparable to DNA sequencing (Tabit 2016).

And as results of this study proved, PCR-RFLP is a powerful method also in the process of SIG discrimination. Due to a high level of biochemical, phenotypic, and sequence similarity (16S rDNA identity is 99.3%) of the SIG, identification of members of this group to the species depends on the sequencing of suitable housekeeping genes. Except for this, also, PCR-RFLP is considered to be a simple and fast method effective in bacteria taxonomy (Olive and Bean 1999; Tabit 2016). PCR-RFLP, a method for identification of *S. intermedius*/*S. pseudintermedius*, was described for the first time by Bannoehr et al. (2009); it is based on the detection of a

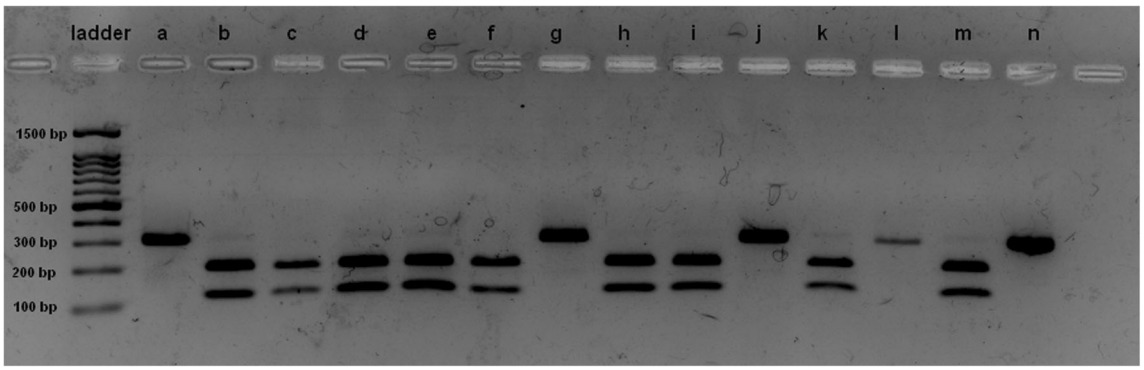


**Fig. 1** Reclassification of putative strains of *Staphylococcus intermedius*/*Staphylococcus pseudintermedius* deposited in the CNCTC. *Staphylococcus pseudintermedius* is characterised by two bands after *Mbo* I digestion. *Staphylococcus intermedius* does not contain a unique restriction site in *pta* gene. Strains: a-5681, b-6050, c-6051, d-6052, e-6053, f-6054, g-6046, h-6047, i-6048, j-6049, k-6718, l-7130, m-7446, n-7634

**Table 2** Biochemical testing of four isolates of *S. intermedius* and type strain of *S. delphini*

| ID of isolate | DNase production | Acetoin production | Trehalose utilisation |
|---|---|---|---|
| 6046 | + | – | + |
| 6049 | + | – | + |
| 7130 | + | – | + |
| 07/514 | + | – | + |
| KTK 28 (SDE)[a] | – | – | – |

[a] Type strain of *S. delphini* (Varaldo et al. 1988; CCM 4115[a] = ATCC 49171[a])

restriction site (↓GATC↑) by *Mbo* I restriction endonuclease in *pta* gene-encoding phosphoacetyltransferase enzyme.

After successful amplification of a 320-bp-long product of *pta* gene from 59 samples of staphylococci, there were two products of 213 and 107 bp in *S. pseudintermedius* and one product (320 bp) in *S. intermedius* detected after cleavage by restriction endonuclease. The results from PCR-RFLP analysis of staphylococci deposited in the CNCTC are given in Fig. 1; gel electrophoresis of the rest of the samples included in this study is not shown. As was proved before, *Mbo* I can detect a restriction site in *S. pseudintermedius*, but not in *S. intermedius* and *S. delphini* (Bannoehr et al. 2009). The lack of restriction site in *pta* gene in both strains (*S. intermedius* and *S. delphini*) is demonstrated by a formation of just one band during gel electrophoresis. Therefore, a complete species identification of four isolates of *S. intermedius* (6046, 6049, 7130, 07/514) was carried out with biochemical testing of a type strain of *S. delphini*. Contrary to *S. delphini*, *S. intermedius* can produce DNase and utilise trehalose (Varaldo et al. 1988; Devriese et al. 2005). Moreover, *S. intermedius* can be distinguishable from *S. delphini* also by an inability to produce of acetoin—VP test (Sasaki et al. 2007). Due to the biochemical testing, no strain of rarely occurring *S. delphini* was detected in this study (Table 2). Our results are not so surprising due to the fact that the occurrence of *S. delphini* is associated mainly with animals like dolphins suffering with skin lesions or sporadically can be detected also in other animals, e.g. horses (Murugaiyan et al. 2014).

From almost all (59/1) examined strains that were originally deposed as *S. intermedius* (or as couple *S. intermedius*/*S. pseudintermedius*), a majority of *S. pseudintermedius* (94%, $n = 53$) was verified and confirmed by comparison with results of Rep-PCR (Švec et al. 2010) (Table 1). Historically, all members of the SIG (*S. intermedius*, *S. pseudintermedius*, and *S. delphini*) were considered as an *S. intermedius* strain. Contrary to the results of Van Hoovels et al. (2006) describing the isolation of *S. pseudintermedius* from humans for the first time, we can confirm that an increasing amount of *S. pseudintermedius* in the population (humans, animals) was a matter of an incorrect classification/misclassification of the SIG in the past. The first isolate of *S. pseudintermedius* from a dog detected in our study was dated to 1985; *S. pseudintermedius* documented as a human pathogen has existed at least early beginning of the 3rd millennium (see Table 1). Our results also indicate that both species have occurred independently for years, having formed individual phylogenetic branches (Bannoehr et al. 2007). Such considerations support the hypothesis about their age-long co-evolution (cf. Fitzgerald 2009).

Despite the fact that *S. pseudintermedius* is a dog's commensal and pathogen, data from literature confirmed a significant prevalence of this species as a human pathogen too (Pompilio et al. 2015; Somayaji et al. 2016). In line with results of other studies, also, we revealed that *S. pseudintermedius* was the most frequent staphylococci species of the SIG found in both humans and animals compared to *S. intermedius* (Devriese et al. 2009; Pompilio et al. 2015; Kmieciak and Szewczyk 2018).

The results of identification of 26 randomly selected staphylococci (from the whole collection of 59 strains) were verified by comparison with results of Rep-PCR analysis (Švec et al. 2010) (Table 1). Positive compliance of results of both methods has proved that PCR-RFLP is a quick method and possesses a suitable discriminatory power for the detection and profiling of bacteria belonging to the SIG. The only limitation of PCR-RFLP method lies in its inability to discriminate between species of *S. intermedius* and *S. delphini*—what can be solved by using additional biochemical tests or by Rep-PCR.

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