Tonsilliphilus suis gen. nov., sp. nov., causing tonsil infections in pigs

Ryozo Azuma,1 Bak Ung-Bok,2 Satoshi Murakami,3 Hiroyuki Ishiwata,4 Makoto Osaki,5 Nobuaki Shimada,5 Yasuichiro Ito,6 Eichi Miyagawa,7 Takashi Makino,8 Takuji Kudo,9 Yoko Takahashi,10 Ikuya Yano,11 Ryo Murata3 and Eiji Yokoyama12

Correspondence
Satoshi Murakami
s1muraka@nodai.ac.jp

12-7-33 Higashi-tokura, Kokubunji-city, Tokyo 185-0002, Japan
21002, 808-dong Suri-Apt, Sanbon-dong, Gunpo-city, Kyong-gi-do, Republic of Korea
3Laboratory of Animal Health, Department of Animal Science, Tokyo University of Agriculture, 1737 Funako Atugi-city, 243-0034, Japan
4Technical Research Institute, Nishimatu Construction Co. Ltd Nakatu, Aikawa-machi Kanagawa-Pref., 243-0303, Japan
5Safety Research team, National Institute of Animal Health, 3-1-5 Kannondai, Tsukuba-city 305-0856, Japan
61-3 Bangai, Tamiya, Ushiku-city, 300-1236, Japan
7Agricultural Microbiology, Faculty of Dairy Science, Rakuno Gakuen University, 582 Bunkyodai, Midorimachi, Ebetu, 069-8501, Japan
8Yakult Central Institute for Microbiological Research 1796 Yaho, Kunitachi-city, 186-8650, Japan
9Japan Collection of Microorganisms, RIKEN BioResource Center, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan
10Kitasato Life Science Institute, Kitasato University, 5-9-1 Shirogane, Minatoku, Tokyo 108-8641, Japan
11Japan BCG Laboratory, 3-1-5 Matuyama, Kiyose, Tokyo 260-8715 Japan

A micro-organism resembling members of the genus Dermatophilus, strain W254T, which was isolated from the submandibular lymph node of a pig, and an additional 16 strains isolated from swine tonsils, were studied to establish their taxonomic status. Although all 17 strains were isolated anaerobically under an atmosphere of 100 % CO₂, all of them were aerotolerant anaerobes. The micro-organisms showed at least five cellular morphologies: (i) a radially protrusive thallus, which proliferated into tuber-like cells; (ii) segmentation in both tubers and thallus followed by multilocule formation, (iii) development of coccoid forms in the locules; (iv) a change from the coccoid forms to zoospores; (v) resting cells, which were able to develop into protrusive thalli again. The micro-organisms were positive for nitrate reduction, but negative for catalase, indole production, hydrolysis of urea and gelatin liquefaction. Milk was not decomposed and none of the strains was haemolytic. A total of 16 compounds, including glucose, were utilized as sole carbon sources and seven compounds, including L-arabinose, were not utilized. Three out of the 17 strains were subjected to further studies. The micro-organisms had meso-diaminopimelic acid in their peptidoglycan and galactose, glucose, madurose and a trace of mannose in their whole-cell sugar patterns. The major phospholipids were phosphatidylglycerol, diphosphatidylglycerol and phosphatidylinositol.

Abbreviations: DAP, diaminopimelic acid; SEM, scanning electron microscopy; TEM, transmission electron microscopy.
The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of Tonsilliphilus suis W254T, HT1-19 and HT5-225 are AB096084, AB096085 and AB602380, respectively.
A supplementary figure and a supplementary table are available with the online version of this paper.
In 1971, a micro-organism resembling members of the genus *Dermatophilus*, strain W254<sup>T</sup>, was isolated from an infected submandibular lymph node of a pig in Japan (Azuma & Bak 1980). In 1978 and 1979, we isolated 10 strains from infected swine tonsils in Korea and their similarity to strain W254<sup>T</sup> was inferred from their morphological resemblance to this strain (Bak & Azuma, 1980, 1991). Six more strains were isolated from swine tonsils in Japan (Azuma et al., 1989). Morphological and physiological studies were performed on all of the isolates and strains W254<sup>T</sup>, HT1-19 and HT5-225 were also subjected to chemotaxonomic and genetic characterization. In parallel with the microbiological characterization, pathology studies on natural and experimentally induced lesions have been conducted (Murakami et al., 1991 and 1992). In this paper, we report the taxonomic characterization of the isolates.

All of the strains were isolated using the gas jet method with MVL medium (Azuma & Suto, 1970; Azuma & Bak, 1980; Azuma & Ito, 1987; Cote, 1992). In some cases a selective medium, MVL containing vancomycin (5 <sup>μ</sup>g ml<sup>−1</sup>), kanamycin (50 <sup>μ</sup>g ml<sup>−1</sup>), nalidixic acid (250 <sup>μ</sup>g ml<sup>−1</sup>) and cephaloridine (3.2 <sup>μ</sup>g ml<sup>−1</sup>) (Azuma et al., 1992), was used. Maintenance cultures of the isolates for inoculating test media were grown on slants of MVL agar for 3–5 days, at 37 °C. For growth under aerobic conditions, brain heart infusion (BHI) or TS (Trypto-soy agar; Eiken) were used. Growth under an atmosphere of 10 % CO<sub>2</sub> was also tested on BHI and TS plates. As indicated above, strain W254<sup>T</sup> was isolated from an infected swine submandibular lymph node in Japan in 1971 and the other strains were isolated from swine tonsils in Korea and Japan from 1978 to 1988. *Dermatophilus congolensis* ATCC 14637<sup>T</sup> was used as a reference strain. TMVL medium, in which 0.1 % Tween 80 is added, was used in place of MVL medium.

Strain W254<sup>T</sup> and selected other strains were examined by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) of ultrathin sections. The isolates were also examined by Gram-, Löffler methylene blue- (Cowan, 1974), lactophenol- (anonymous 1989) and flagellar-staining (Research Group 1951). For SEM, the specimens were fixed with both 2 % glutaraldehyde and 1 % osmium oxide, then dehydrated, critical point dried, coated with Palladium–Platinum and viewed under a Hitachi S-450 electron microscope. For TEM, the same double fixation was used as for SEM and the specimens were embedded in Epon 812. The resulting blocks were sectioned and the sections were stained with uranyl acetate and lead acetate. The specimens were examined under a Hitachi EU-12 electron microscope.

Five per cent sheep blood BHI agar for haemolysis; a TMVL or BHI culture for catalase; 0.1 % KNO<sub>3</sub> BHI broth for nitrate reduction; 1 % yeast extract Trypticase (BBL) broth for indole production and SSR (Stuart, van Stratum & Rustigan) medium for urea hydrolysis, were used (Cowan, 1974). Gelatin liquefaction was performed using a denatured gelatin block (Kohn, 1953). Skim-milk (10 %) added to BHI was used for the milk decomposition test. For sugar utilization, basal semi-solid medium consisting of polypeptone (Daigo) 1 %, yeast extract (Difco) 0.2 %, NaCl 0.5 %, Andrade’s indicator (1 ml) and bactoagar (Difco) 0.2 % was used. Each sugar (10 % w/v) was sterilized by autoclaving with the exception of L-arabinose, xylose and ribose which were filter-sterilized. All carbon sources were added to the basal medium at a final concentration of 1 % (w/v). Sugars used are indicated in Table S1 (available in IJSEM Online).

The isomer of diaminopimelic acid (DAP) in the peptidoglycan was determined as described by Azuma et al. (2009). A standard amino acid mixture solution (Type H, Wako Pure Chemical Industries) and a mixture prepared from DAP, ornithine–HCl and N-acetyl muramic acid (all from Wako Pure Chemical Industries) were used as standards. Whole cell sugars were determined by the method of Staneck & Roberts (1974) using descending paper chromatography. Phospholipids were determined by the method of Folch et al. (1957). For detection of phosphorus, amino groups and reducing sugars, the Dittmer, ninhydrin and diphenylamine reagents were used, respectively. As standards, diphostatidylglycerol (A42, Funakoshi), phosphatidyglycerol (A431, Funakoshi) and phosphatidylinositol (A39, Funakoshi) were applied to the same plate. Cellular fatty acids were determined as described by Azuma et al. (1992) for strains W254<sup>T</sup>, HT1-19, HT5-225 and *D. congolensis* ATCC 14637<sup>T</sup> grown in TS broth (Rohde, 1973) at 37 °C for 5 days. The fatty acids were analysed using a model 163 gas chromatograph (Hitachi) equipped with a PEG-HT column, (0.25 mm internal diameter × 50 m; GL Science). A M-80B double focusing mass spectrometer (Hitachi) was also used for the
confirmation of some fatty acids. Menaquinones were determined by the method of Collins et al. (1977) using an HPLC (LC 6A; Shimadzu) with an Ultron S-C_{18} column (Chromato Pack) and a mass spectrometer (QP-1000). The G+C content of the DNA was determined according to the method of Ezaki et al. (1990).

DNA–DNA hybridization experiments were performed according to the method of Tamura et al. (2000). Strains W254\textsuperscript{T}, HT1-19 and HT5-225 were cultured at 37 °C for 1 week in TMVL broth and their DNAs were extracted. Calf thymus DNA was used as a negative control.

Genomic DNA was used for amplification of the 16S rRNA gene as described by Saiki et al. (1988). The amplification was carried out with a TaKaRa PCR amplification kit. For cloning, the PCR products were ligated into the PGEM-T vector (Promega) using the TaKaRa ligation kit ver. 2. Plasmid purification from Escherichia coli strain JM109 was performed with a Qiagen plasmid mini kit. Five clones were pooled for the sequencing reaction in order to reduce possible errors due to the Taq DNA polymerase. The DNA sequences of cloned fragments from strains W254\textsuperscript{T} and HT1-19 and HT5-225 were determined by using an Applied Biosystems model 377 DNA sequencer and big dye primer cycle sequencing kit (Applied Biosystems) according to the manufacturer’s directions. Sequence analysis was outsourced to Greiner Japan (Tokyo).

The 16S rRNA gene sequences of strains W254\textsuperscript{T}, HT1-19 and HT5-225 were investigated with those of other bacteria of the suborder Micrococccineae (Schumann et al., 2009) using MEGA software version 5 (Tamura et al., 2011). All the sequences registered in GenBank were retrieved from positions equivalent to positions 41 to 1475 of the sequence of Escherichia coli (accession number J01695) and subjected to analysis. Alignment of these sequences was carried out using CLUSTAL W and phylogenetic trees were reconstructed by maximum-likelihood and maximum-parsimony methods using MEGA version 5. Bootstrap values were determined based on 1000 replications.

The 17 strains examined were isolated anaerobically under 100 % CO\textsubscript{2}. Cultures on BHI under 100 % CO\textsubscript{2} grew better than those incubated under aerobic conditions. The growth of cultures of D. congolensis ATCC 14637\textsuperscript{T} incubated under 100 % CO\textsubscript{2} was distinctly poorer than that of those incubated under aerobic conditions. Incubation of the isolates on agar slants under 100 % CO\textsubscript{2} resulted in dense and granular colonies or irregular, rough colonies of various sizes. However, under aerobic conditions the colonies were larger, harder and had various shapes (e.g.

Fig. 1. (a) *Tonsilliphilus suis* sp. nov. strain 79, 12-1 grown on BHI agar in 10 % CO\textsubscript{2} for 11 days at 37 °C. Prepared by the lactophenol method. A radiating segmented thallus in an early stage of growth. Bar, 10 μm. (b) Strain 79, 12-1 grown on BHI agar in 10 % CO\textsubscript{2} for 11 days at 37 °C. Prepared by the lactophenol method. A chlamydospore on a mature thallus is shown (indicated with an arrow). Bar, 10 μm. (c) Light micrograph of *Tonsilliphilus suis* sp. nov. strain 79, 3-1 grown on BHI agar in 10 % CO\textsubscript{2} for 8 days at 37 °C. Prepared using Loffler’s methylene blue staining method. A thallus is shown in which segmentation is visible as well as coccoid forms in locules. Inset: a chlamydospore (arrow) is shown. Bar, 500 nm. (d) Transmission electron micrograph of strain W254\textsuperscript{T} grown on a MVL slant in 100 % CO\textsubscript{2} for 5 days at 37 °C showing a cross section through a tuber. The development of coccoid forms in multilocules can be seen. Bar, 10 μm. (e) Strain 79, 12-1 grown on TS agar in 10 % CO\textsubscript{2} for 3 days at 37 °C showing zoospores with flagella. Prepared by the flagellar staining method. Bar, 10 μm.
umbonate and molar tooth colonies). On occasion, whole colonies were covered with a slimy substance.

The dominant cellular morphology was short radial protrusions or thalli (Fig. 1a). The thalli developed into tubers, by growing wider (Fig. 1b). Horizontal and vertical segmentation occurred in the tubers and thalli, which led to formation of multi-loculi (Fig. 1c) in which coccoid cells later appeared (Fig. 1d). A single flagellated zoospore (Fig. 1e) emerged from each mature locule. Each zoospore germinated to become a resting cell. Budding of the resting cells led to the development of a young thallus. Sometimes chlamydospores were seen (Figs 1b, d).

All of the strains were positive for nitrate reduction, but negative for catalase, indole, urea hydrolysis, gelatin liquefaction, milk decomposition and haemolysis. D. congolensis ATCC 14637T produced the opposite result for each of these tests (except indole production). The pig isolates utilized 16 of 26 compounds tested as sole carbon sources. Seven compounds were not utilized and the results for ribose, cellobiose and soluble starch varied between the strains. D. congolensis ATCC 14637T utilized only four sugars (Table S1). Thus the pig isolates are saccharolytic, whereas D. congolensis ATCC 14637T is proteolytic.

Chemotaxonomic characteristics are shown in Table 1. The isomer of DAP, the phospholipid pattern (Azuma et al., 1989) and the major menaquinone of the novel strains were the same as those of D. congolensis ATCC 14637T. However, the whole cell sugar pattern of the novel strains contained four sugars, including madurose (Azuma et al., 1989), whereas D. congolensis ATCC 14637T contains only madurose.

The cellular fatty acid profiles of the novel strains and D. congolensis ATCC 14637T were different, with those of the novel strains containing fewer fatty acids. The difference in the DNA G+C content of the novel strains and D. congolensis ATCC 14637T was marked.

The homologous reassociation values between labelled DNA of strain W254T and unlabelled DNA of strains HT5-225 and HT1-19 were 82.9 % and 86.4 %, respectively. The DNA relatedness values between labelled DNA of strain HT5-225 and unlabelled DNA of strains W254T and HT1-19 were 85.2 % and 87.5 %, respectively. The DNA relatedness values between labelled DNA of strain HT1-19 and unlabelled DNA of strains W254T and HT5-225 were 88.3 % and 89.4 %, respectively. The levels of DNA relatedness for the three strains did not fall below 85 %.

Table 1. Chemotaxonomic characteristics that differentiate strains W254T, HT1-19 and HT5-225 from their close relatives

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Whole-cell sugars</strong></td>
<td>Galactose, glucose, mannose, madurose</td>
<td>Galactose, glucose, mannose, madurose</td>
<td>Galactose, glucose, mannose, madurose</td>
<td>Madurose</td>
<td>Ribose, Mannose</td>
</tr>
<tr>
<td><strong>Phospholipids</strong></td>
<td>Phosphatidylinositol, phosphatidylglycerol, diposphatidylglycerol</td>
<td>Phosphatidylinositol, phosphatidylglycerol, diposphatidylglycerol</td>
<td>Phosphatidylinositol, phosphatidylglycerol, diposphatidylglycerol</td>
<td>Phosphatidylinositol, phosphatidylglycerol, diposphatidylglycerol</td>
<td>Phosphatidylglycerol, Phosphatidylinositol lyso-Phosphatidylethanolamine</td>
</tr>
<tr>
<td><strong>Cellular fatty acids (%)</strong></td>
<td>23.3</td>
<td>23.1</td>
<td>35.7</td>
<td>30.0</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>Un.</td>
<td></td>
<td></td>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td>C13 : 0</td>
<td>2.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14 : 0</td>
<td>1.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C15 : 0</td>
<td>9.4</td>
<td>1.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Un.</td>
<td></td>
<td>1.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16 : 0</td>
<td>5.9</td>
<td>2.9</td>
<td>2.4</td>
<td>16.7</td>
<td>7.4</td>
</tr>
<tr>
<td>C17 : 0</td>
<td>39.5</td>
<td>62.9</td>
<td>45.6</td>
<td>12.3</td>
<td>6.0</td>
</tr>
<tr>
<td>C17 : 1</td>
<td>24.4</td>
<td>2.3</td>
<td>1.6</td>
<td>16.7</td>
<td>23.9</td>
</tr>
<tr>
<td>C18 : 0</td>
<td></td>
<td></td>
<td>4.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Menaquinone</td>
<td>MK-8(H4)</td>
<td>MK-8(H4)</td>
<td>MK-8(H4)</td>
<td>MK-8(H4)</td>
<td>MK-8(H4)</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>72.2</td>
<td>67.2</td>
<td>71.5</td>
<td>58.99</td>
<td>71.5</td>
</tr>
</tbody>
</table>

*P. intestinalis Ngc37-23T also contains major amounts (>10 %) of iso-C14 : 0 and iso-C16 : 0.
†Data taken from Hamada et al. (2010).
80 % for any pair. By convention, strains which exhibit more than 70 % DNA relatedness are considered members of the same genomic species (Wayne et al., 1987).

When the common regions of the 16S rRNA gene sequences of strains W254T (1470 bp), HT1-19 (1517 bp) and HT5-225 (1480 bp) were analysed, only one base was different in HT1-19, indicating that the similarity between these three strains was more than 99 %. Comparative sequence analysis showed that the 16S rRNA gene sequence similarity between strain W254T and *Dermatophilus congolensis* ATCC 14637T was 94.7 %. The maximum-likelihood phylogenetic tree (Fig. 2) and maximum-parsimony phylogenetic tree (Fig. S1) clearly revealed that strains W254T, HT1-19 and HT5-225 formed a cluster with the type strains of bacteria in the family *Dermatophilaceae*, indicating that these three strains belong to this family.

Strain W254T was sent to Dr H.A. Lechevalier in 1984. He informed us of the presence of meso-DAP and madurose (Personal communication, 1984). *Tonsillophilus suis* was subsequently included in Bergey’s manual (Gordon, 1989). Although strain W254T was isolated from a submandibular lymph node, the other 16 strains were all isolated from swine tonsils (Azuma et al., 1990). From this, it is possible to suggest that the habitat of the isolates might be infected swine tonsils, and by chance the organisms invade neighbouring lymph nodes.

By cooperative pathological research, it became clear that the micro-organisms might preferably infect the swine

![Fig. 2. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences (1435 nt). The sequence of *Escherichia coli* (J01695) was used as an outgroup. Numbers at nodes indicate percentage bootstrap support based on a maximum-likelihood analysis of 1000 resampled datasets. Bar, 5 nt substitutions per 100 nt.](http://ijs.sgmjournals.org)
tonsillar crypt (Bak & Azuma, 1991; Murakami et al., 1991). Also, it was revealed that the incidence of tonsil infection reached 10.5–57.1% in fattening pigs (Bak & Azuma, 1980; Murakami et al., 1991) and breeding sows (Shiozawa et al., 1991). With regard to the infection of the swine tonsillar crypt, Johne (1881, 1882) described the involvement of a fungus of the genus *Echinobotryum*. The organism sketched by him appears to be similar to one of the novel isolates. Gilka (1983) observed club-forming micro-organisms in swine tonsillar crypts by electron microscopy. Experimental infection using the novel isolates in mice (Momotani et al., 1983), rats, guinea pigs (Murakami et al., 1992) and sheep (Momotani et al., 1985) were performed, in which some pathogenic potential of the organisms was shown.

Comparative infectivity of the novel isolates and that of *D. congolensis* (Gordon, 1964) should be mentioned. The novel isolates were associated with swine tonsillar crypts, while *D. congolensis* is associated with bovine hair follicles (Abu-Samra et al., 1976; Kinjo et al., 1981), which are anatomically similar to tonsillar crypts. In both species, adaptive features of the micro-organisms to host animals, that is, microbial elements, histological constituents and the process of lesion development seem very similar. The novel isolates seem to have weak infectivity, because lesions are confined to the tonsil itself, although spread from one crypt to a neighbouring crypt or those of another host are suspected. *D. congolensis* has a similar infection pattern and hair follicles in the skin of cattle are infected. The described cases give the impression of serious illness, although some bad climatic or feeding conditions might also have been involved (Hyslop, 1979). One reason is the fact that *D. congolensis* has proteolytic and haemolytic ability. From the above consideration, the virulence of *D. congolensis* might be higher than that of the novel isolates.

Other actinobacteria have been reported from swine tonsils. The presence of species of the genus *Actinomyces* in swine tonsils was reported by Johne (1881), Davis (1923) and Ogura (1925), and *‘Actinomyces suis’* was isolated from swine tonsils/the swine oral cavity (Oomi et al., 1994). Subsequently, the presence of the bacteria in swine tonsils has been demonstrated by pathological and immunohistochemical studies (Murakami et al., 1997, 1998).

### Description of Tonsilliphilus suis gen. nov.

*Tonsilliphilus* [Ton.sil.li phi’lus. L. pl. n. *tonsilla* the tonsils in the throat; N.L. masc. n. *philus* (from Gr. masc. n. *philos*) a friend; N.L. masc. n. *Tonsilliphilus* a friend of tonsils].

Gram-positive, aerotolerant anaerobes. The peptidoglycan contains meso-DAP as the characteristic diaminoc acid and the whole-cell sugars are galactose, glucose and madurose with traces of mannose. The major phospholipids are phosphatidylinositol, phosphatidylethanolamine and diphosphatidylglycerol. The cellular fatty acids are C15:0, C16:0, C17:0 and C18:0 (C17:1 may be present). The major menaquinone is MK-8(H4). The genus belongs to the family *Dermatophilaceae*. The type species is *Tonsilliphilus suis*.

### Description of Tonsilliphilus suis sp. nov.

*Tonsilliphilus suis* (su’is. L. gen. n. suis of a pig).

General chemotaxonomic characteristics are as described in the genus description. Cells show stronger growth under 100% CO2 than under aerobic conditions. Cells develop into a short radial protrusion or thallus, which then develops into tubers. In both thalli and tubers, horizontal and vertical segmentation occurs to form multi-loculi. Thereafter, coccoid cells develop in the multi-loculi. A single flagellated zoospore emerges from each mature locule. The zoospore germinates to become a resting cell. Budding of the resting cells leads to the development of a new thallus. Globose bodies or chlamydospores are sometimes formed. Colonies are dense and granular or irregular and rough on the surface after growth on TMVL slants in 100% CO2 after 3–5 days at 37°C. Positive for nitrate reduction but negative for catalase, indole production, urease, gelatin liquefaction and milk decomposition. Not haemolytic. Aesculin, fructose, galactose, glucose, inositol, inulin, lactose, maltose, mannitol, mannose, melibiose, raffinose, salicin, sorbitol, sucrose and trehalose are utilized as sole carbon sources, but adonitol, l-arabinose, dulcitol, glycerin, melzezitose, rhamnose and xylose are not used. The utilization of cellobiose, ribose and starch varies between strains. The G+C content of the DNA is 69.6–71.8 mol%. Most strains have been isolated from inflamed swine tonsillar crypts.

The type strain, W254T (=ATCC 35846T=CCM 3774T=DSM 21880T=JCM 15727T), was isolated from an infected swine submandibular lymph node.

### Acknowledgements

The authors express sincere gratitude to Y. Okada, F. Yoshikawa, S. Ikemoto M. Shiozawa, Dr E. Momotani, Dr H. Fujiwara, Dr H. Oomi, Dr T. Watanabe, Dr S. Suzuki, the late Dr N. Hara, the late Dr S. Inui and the late T. Koeda for their informative and technical cooperation.

### References


