Oral colonization of aerobic and facultatively anaerobic gram-negative rods and yeast in Tibetans living in Lhasa.

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Running title: Oral coliforms and yeasts in Tibetans

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Abstract

This study aimed at investigating the oral colonization of aerobic and facultatively anaerobic gram-negative rods and yeasts in Tibetans living in Lhasa, Tibet Autonomous Region, China. Samples of children (n = 50) and adults (n = 38) were selected from pools of 207 children, (11 – 13 year-olds; from 2 primary schools) and 94 adults (25 – 44 year-olds; from 4 governmental agencies) who were the subjects for an oral health survey. Mean ages for the study group of children (38% female) and adults (61% female) were 11.6±0.9 years and 37.1±6.1 years, respectively. All subjects had lived in Tibet since birth. Selective culture of oral rinse samples was carried out to isolate, quantify and speciate aerobic and facultatively anaerobic gram-negative rods (using API 20E kit) and yeast (using API 20C AUX and API ZYM kits). Oral coliform bacteria and yeast isolation rates for children were 84% and 14%, respectively while the respective rates for adults were 26% and 40%. The corresponding quantity of coliforms/yeasts for children and adults were $0.4\pm1.6\times10^3$ cfu/15.8±72.3 cfu and $0.2\pm0.6\times10^3$ cfu/57.2±137.5 cfu per ml oral rinse, respectively. Recovery of aerobic and facultatively anaerobic gram-negative rods and *Stenotrophomonas maltophilia*, a free-living saprophytic and ubiquitous bacterial species of wide geographic distribution, in the oral rinse samples was found to be significantly more prevalent in children. The isolation rates of facultatively anaerobic gram-negative rods in adults and yeasts in both groups were compatible with previous similar cohorts from southern China. Randomly amplified polymeric DNA analysis showed that the *S. maltophilia* species isolated from children were of several different clonal types and were school specific. This study showed that while the colonization rate of facultatively anaerobic gram-negative rods in adults and yeasts in both groups were similar to populations at lower altitudes, the native young, urban Tibetans appear to exhibit a high oral carriage rate of *S. maltophilia* species. The possible nature and implications of this high prevalence of *S. maltophilia* are discussed.

Keywords: Enteric rods; Oral microbiology; *Stenotrophomonas maltophilia*; Tibetans; yeasts

1. Introduction

Tibetans are one of few tribal groups in the World that reside at high altitudes. Little information on their oral health status or their oral microflora is available. Recently, we conducted an oral health status survey of inhabitants of Lhasa and found that in children, the treatment need for dental caries was low while their periodontal health status was unsatisfactory (Lo et al., 2000). The periodontal health status of the Lhasa adults surveyed was also considered to be unsatisfactory (Corbet et al., 2000). Similar oral health conditions were detected between the native Tibetans and Han Chinese living in Lhasa. However, no information is available in the literature relating the impact of unique living environment, life-style, and poor periodontal health to the human oral microbiology. The present oral microbial project was hence conducted to investigate the colonization pattern of aerobic and facultatively anaerobic gram-negative rods and oral yeasts in Tibetans living in Lhasa. The hypothesis of the current study was that the environmental influence of reduced atmospheric oxygen tension at high attitudes as well as the special high protein low vegetable diet of Tibetans might affect human oral microbial ecology.

Stenotrophomonas maltophilia is a free-living saprophytic and ubiquitous bacterial species of wide geographic distribution. It is a short to medium-sized straight gram-negative rod with a polar tuft of flagella (von Graevenitz, 1995). It was previously named as *Pseudomonas maltophilia* (Hugh 1981) and then was transferred to *Xanthomonas maltophilia* (Swings et al., 1983) followed by its current new genus and name for taxonomical reasons (Palleroni and Bradbury, 1993). *S. maltophilia* is also commonly isolated from the hospital environment. It is the third most frequently isolated nonfermentative gram-negative rod in the clinical laboratory and is frequently resistant to antimicrobial agents (von Granevenitz, 1995).

2. Materials and Methods

2.1 Subjects

A total of 50 Tibetan children and 38 Tibetan adults were selected from two pools of 207 children and 94 adults attending a survey conducted by the study group in Lhasa city, Tibet Autonomous Region, China. The 11-13 year-old children recruited were from the two biggest primary schools in the city (Lo et al., 2000) while the adults were recruited from four local governmental agencies. All survey sites were situated at the city centre. To make sure that the samples did not go through undue storage before processing, only subjects who participated in the survey during the last 2 days of its conduct were recruited.

2.2 Sampling

Oral rinse sampling was carried out as per (Leung et al., 2000). In brief, all subjects were asked to rinse their mouth for 60s with 10 ml of sterile phosphate buffered saline, pH 7.2 (PBS). Denture-wearing subjects did not remove their prosthesis. After 60s the subjects expectorated the oral rinse into a sterile universal container, which was then transferred into a 13 ml sterile screw-cap centrifuge tube for storage at 4^{o}C before laboratory processing. All samples were immediately shipped to Hong Kong after the survey concluded and were processed within 72 hours after sampling.

2.3 Culture

All samples were centrifuged at 1700 x *g*, 10 min. The pellet was resuspended in 2.5 ml of PBS and vortexed for 30s at maximum setting (Autovortex Mixer SA2, Stuart Scientific, London, UK). Fifty μl of the resuspended oral rinses were spiral plated (Model DU, Spiral Systems, Cincinnati, OH) onto duplicate MacConkey agar and Sabouraud's dextrose agar (Oxoid, Hampshire, UK) plates and incubated for 18h at 37^{o}C. Afterwards all

cultures were examined and the colony-forming units of coliform and yeasts in each sample were quantified. The aerobic and facultatively anaerobic gram-negative rods and yeasts were then sub-cultured to obtain pure isolates.

2.4 Identification of isolates

Pure colonies of aerobic and facultatively anaerobic gram-negative rods were identified presumptively on the following features: Colony morphology, colonial pigmentation, cell morphology, Gram-staining reaction, oxidase positively. Isolates were further characterized biochemically using the API 20E Kit (Analytical Profile Index, Bio Mérieux SA, France). Pure colonies of yeasts were identified and speciated based on the following: colony morphology, cell morphology, Gram-staining reaction, germ tube test and characterization by API 20C AUX and API ZYM kits (Leung et al., 2000).

2.5 Stenotrophomonas maltophilia *DNA extraction and preparation for randomly amplified polymorphic DNA analysis.*

A follow-up investigation was undertaken in view of the observation of a considerably high prevalence of *S. maltophilia* isolated from the oral rinse samples of Tibetan children. Isolates were grown overnight at 37ºC on Mueller – Hinton ager (Oxiod, Hampshire, UK). Genomic DNA were extracted from the *S. maltophilia* using the Wizard® Genomic DNA purification kit (Promega, Madison, USA). DNA yield and integrity were verified through electrophoresis in 1% agarose gel. For randomly amplified polymorphic DNA (RAPD) analysis, the PCR protocol reported by Davin-Regli and co-workers (1996) was used with slight modifications. The custom-synthesized primers (Gibco BRL; Hong Kong) used in the study were AP4 (5'TCACGATGCA3') and AP12H (5'CGGCCCCTGT3') (Williams et al., 1990). The other two primers, i.e. AP13 and AP12R used by the French

group (Davin-Regli et al., 1996) were also tried, but failed to provide easily discernable banding patterns. The corresponding data were thus not included in this report. Thermocycling was performed in a GeneAmp 9700 machine (Perkin Elmer, Foster City, USA). A 50 μl volume of the PCR master mix contained approximately 50-100 ng of *S. maltophilia* DNA template, 5 μl of PCR buffer [10 x PCR buffer: 0.5M KCl-25mM $MgCl_2$-1% Triton X-100 (v/v) – 0.2 MTris (pH 8.4)], a 200 μM concentration of each dNTP, an 1 μM concentration of primer, and 1.5U of *Taq* polymerase (Life Technologies, Frederick, Md). The machine was programmed for 45 cycles of 60s denaturation at 94^{o}C and 2 min of annealing at 46^{o}C and 2 min of primer extension at 72^{o}C. The reaction was held at 72^{o}C for 10 min at the end. Control tubes without template DNA were included in each run, and reproducibility was checked for each reaction (Davin-Regli et al., 1996). The PCR products were electrophoresed in a 1% agarose gel in TBE buffer, stained with ethidium bromide, and visualized under UV transillumination.

2.6 Data analysis and statistics

The demographic and microbiological data of the subjects were analyzed by Statview 4.5 for Macintosh. Differences between individual groups were tested by analysis of variance (ANOVA), or Fisher exact test as appropriate. Groups were regarded as significantly different from each other if P was < 0.05.

RAPD patterns of each *S. maltophilia* isolate were analyzed with Dendron® 3.0 Programme (Solltech, Oakdale, USA) The unweighted pair group method with arithmetic means (UPGMA method) was used for clustering of the isolates on the dendrogram. Closely related genetic clusters of *S. maltophilia* were identified and distribution of such *S. maltophilia* clonal types among the two schools surveyed (i.e. School A and B) was analyzed.

3. Results

3.1 Demography

The demography of the recruits are as follows. Mean age of the child recruits was 11.6±0.9 yr while the mean age of the adult recruits was 37.1±6.1 yr (20-46 yr). There were 19 (38%) girls in the children's group and 23 (61%) females in the adult group.

3.2 Microbial Culture

The individual microbial families, their number and the identity of aerobic and facultatively anaerobic gram-negative rods and yeasts retrievable from oral rinse samples of each individual groups are recorded in Table 1. Lowest detection limit of the oral rinse technique was: 5 colony-forming units/ml oral rinse. The mean number of aerobic and facultatively anaerobic gram-negative rods species isolated from the Children/Adult groups were 0.9 ± 0.5 / 0.4 ± 0.8 respectively. Oral colonization by aerobic and facultatively anaerobic gram-negative rods in Tibetan children was highly prevalent (84%) and was almost exclusively by a member of the *Pseudomonadaceae* family, i.e. the species *S. maltophilia* (Table 1). The adult group, however, did not show preferred colonization by any particular aerobic and facultatively anaerobic gram-negative rods species. Significant differences were detected between children and adult groups in mean colony forming units of aerobic and facultatively anaerobic gram-negative rods' families recovered and the corresponding prevalence (*Pseudomonadaceae/Enterobacteriaceae:* 0.8 ± 0.4 / 0.0 ± 0.1 vs 0.0 ± 0.3 / 0.3 ± 0.7 in children vs adult group respectively, $P < 0.03$, ANOVA). On the other hand, the overall prevalence of yeasts were found to be significantly higher in adults than in children (Table 1).

Further quantitative (colony-forming units/ml) data related to the aerobic and facultatively anaerobic gram-negative rods and yeasts recovered from the oral rinse samples

are summarized in Table 2. A remarkable range in the quantity of *S. maltophilia* recovered from the oral rinse samples from children was noted. However, no significant difference in terms of the various bacterial or yeast species isolated could be detected between the children and the adults group. Also, no significant difference was noted in the quantity of *S. maltophilia* colonizing children from the two schools surveyed (medians of 84 vs 122 colony-forming units/ml).

3.3 RAPD analysis of S. maltophilia *isolates*

Representative RAPD patterns of the 64 (0-3 isolates per person, 1.3±0.8 isolates per oral rinse sample) *S. maltophilia* isolates using primers AP4 and AP12H are shown in Figure 1. Primers AP4 and AP12H each produced distinct clonal group patterns from the isolates. Based on the RAPD results, dendrograms were generated from data set on each RAPD primer. A S_{AB} 0.75 threshold was selected to subgroup closely related clusters (primer AP4: subgroups A, B & C; primer AP12H: subgroups I, II, III, IV and V) of isolates. The RAPD results generated from primers AP4 and AP12H, i.e. the various subgroups, were then combined in the subsequent analysis which resulted in nine colonal types of *S. maltophilia* being identified (i.e. AI, AII, AIII, AIV, BI, BII, BIII, BIV and CV). A total of 19 strains, however, were of S_{AB} values > 0.75 and were put under "others" category (Table 3). In four of the oral rinse samples, the same *S. maltophilia* clonal type was identified from two isolates and they were hence considered as one count, resulting in 58 specimens to be analyzed (Table 3). Children from one school (School A) were found to be colonized by seven identifiable *S. maltophilia* clonal types, predominantly AIII (20% of the children in that school were positive for this clonal type) and BIV (13%) while children from the other school were colonized by five identifiable *S. maltophilia* clonal types with type AII (46%) being the dominant colonizer and was significantly more prevalent in school B (Table 3). Considering

all data together, it appeared from the analysis that the genetic pattern/identity of *S. maltophilia* isolated from Tibetan children attending these two schools were different.

4. Discussion

It was shown by Sedgley and co-workers that enteric gram-negative rods, especially *Enterobacteriaceae* are relatively more common in Southern Chinese (exclusively of the Han ethnic group), while the corresponding oral yeast prevalence compares favourably with reports from other parts of the world (Sedgley and Samaranayake, 1994, Sedgley et al., 1997). On the other hand, increase in oral prevalence of aerobic and facultatively anaerobic gram-negative rods and yeasts with increasing age (Sedgley and Samaranayake, 1994), hospitalization (Sedgley et al., 1995) and medical conditions leading to xerostomia (Leung et al., 2000, Leung et al., 2001) is well established in the literature. The present study attempts to investigate the colonization patterns of aerobic and facultatively anaerobic gram-negative rods and yeasts in a special ethnic group (Tibetans) living in a high altitude in China.

Lhasa, the capital city of the Tibet autonomous region, is at 3,658m above sea level with a population of about 300,000. Our previous reports about the same population studied had shown that dental caries and caries treatment need level of both Tibetan and Han children in Lhasa were equally low but that periodontal conditions were not satisfactory (Lo et al., 2000). Tibetan and Han adults living in Lhasa had similar oral health conditions. They exhibit a mean DMFT of 4 and 70% with a need for caries treatments, none having healthy periodontal conditions, about 50% having calculus but no pockets and 16% having deep pockets (Corbet et al., 2001). Such periodontal disease distribution is similar to that of Hong Kong (Holmgren et al., 1994).

While the oral gram-negative enteric rod prevalence of the adult Tibetan surveyed is comparable to Southern Chinese (Sedgley and Samaranayake, 1994, Sedgley et al., 1997), the

present study, in contrast to the previous report (Sedgley et al., 1997) demonstrated a very high prevalence of *S. maltophilia* isolation (Table 2) in Tibetan children [82% vs 1% reported by Sedgley and co-workers (Sedgley et al., 1997)]. The high oral colonization by *S. maltophilia* in the child cohort was remarkable. Only one out of all 42 children who were colonized with oral aerobic and facultatively anaeobic gram-negative rods was inhabited by a coliform other than *S. maltophilia*. Follow up investigation was conducted after such observation to further characterize the colonization pattern of *S. maltophilia* in the Tibetan children surveyed.

S. maltophilia is of wide geographic distribution and is usually harmless for healthy individuals (Davin-Regli et al., 1996, Palleroni and Bradbury, 1993). The quantity of *S. maltophilia* detected from the oral rise samples of the healthy children surveyed was 10-10,000 times less than the reported oral infectious doses (Rusin et al., 1997) and hence no subject seemed to be overly affected by this 'resident' coliform. Another feature of *S. maltophilia* that might be of medical relevance is that they are naturally resistant to most broad-spectrum antimicrobial agents and hence is emerging as a rather troublesome nosocomial pathogen (Denton and Kerr, 1998, Higgins et al., 2001, Penzak and Abate, 1997).

The fact that oral prevalence and the profile of aerobic and facultatively anaerobic gram-negative rods colonization in Tibetan adults were comparable to the Southern Chinese (Sedgley and Samaranayake, 1994) indicates that the oral *S. maltophilia* colonization observation in the present study is probably children specific. In other words, the oral aerobic and facultatively anaerobic rods colonization in Chinese adults, seems not to be affected by ethnicity, altitude or diet. The exact reason for the high prevalence of oral *S. maltophilia* colonization in Tibetan child is unknown. Nine major clonal types of *S. maltophilia* were identified using the RAPD in combination with the UPGMA clustering analysis. These nine clonal types were not randomly distributed among the children from the

two primary schools studied. The fact that multiple and different clonal types of *S. maltophilia* were isolated from the two schools (7/9 from one and 5/9 from the other) indicates that the oral "infection" of *S. maltophilia* was not from a major single reservoir e.g. from contaminated drinking water, food etc. If that were to be the case, colonization by single major clonal type of *S. maltophilia* would be expected. A possible explanation for this multiclonality seen only in children could be the acquisition of multiple free living *S. maltophilia* stains from the environment, for instance from the school playgrounds. From the foregoing observation it is apparent that provided the appropriate conditions are satisfied, aerobic and facultatively anaerobic rods, in this case *S. maltophilia*, could colonize the human mouth with relative ease. It therefore follows that such bacteria could also colonize oral cavities of sick or malnourished hosts with same efficiency leading to serious medical outcomes (Rusin et al., 1997)). Due to the pressure from other workload and the field working environment, the research team had to focus this microbiological study on Tibetans and samples from the Han cohort who took part in the oral health survey were not taken. For comparison, further studies on the oral aerobic and facultatively anaerobic gram-negative rods carriage in Tibetan and Han children under the same environmental conditions are warranted to investigate any ethnic influences.

Our data on oral carriage of yeasts in urban Tibetan children and adults is similar to reports from the Southern Chinese of Hong Kong (Sedgley and Samaranayake, 1994, Sedgley et al., 1997). The most common yeast isolated was *Candida albicans*. Other yeast species identified were *C. tropicalis*, *C. guilliermondii* and *C. parapsilosis.*

In conclusion, the current study demonstrates that native urban Tibetans like their Southern Chinese counter-parts also harbour aerobic and facultatively anaerobic gram-negative rods and yeasts in their oral cavities indicaitng the reduced atmosphere oxygen tension at high altitudes and their life-style in terms of diet did not seem to have influenced

the oral microbial ecology of the subjects surveyed. An unexpected high prevalence of multiclonal *S. maltophilia* colonization was found among the Tibetan school children and the colonization by clonal types of the enteric rod seemed to be school specific. *S. maltophilia* is known to possess multi-antimicrobiol resistant determinants (Kelly and Mortensen 1995, Penzak and Abate, 1997, Poole, 2001) thus the relevance of its high colonization in Tibetan children is worthy of further study. The material presented in this report arises from a convenience sample, however it is the first study investigating the oral aerobic and facultively anaeobic gram-negative rods and yeasts colonization in Tibetans' mouth. More comprehensive longitudinal investigations with a larger test and control groups with follow up sampling are required to confirm our preliminary findings.

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Figure Captions

Fig. 1. Typical RAPD profiles of different clonal groups of *Stenotrophononas maltophilia* generated with primers (A) AP4 (5'TCACGATGCA3') and (B) AP12H (5'CGGCCCCTGT3'). Lanes: M, DNA Ladder Mix (MBI Fermentas); **1**, isolate 5084 [clonal type AI]; **2**, isolate 6007a [clonal type AII]; **3**, isolate 5037a [clonal type AIII]; **4**, isolate 5079a [clonal type AIV]; **5**, isolate 5085a [clonal type BI]; **6**, isolate 6013b [clonal type BII]; **7**, isolate 5106b [clonal type BIII]; **8**, isolate 6013a [clonal type BIV]; **9**, isolate 5022 [clonal type CV]. Electrophoresis was carried out in a 1% agarose gel.

Table 1.
Percentage frequency of aerobic and facultatively anaerobic gram-negative rods species and yeasts isolated from oral rinse samples[a].

	Child	Adult
***Pseudomonadaceae*[b]**	**82.0**	**13.2**
Pseudomonas fluorescens/putida	0	5.3
Pseudomonas paucimobilis	0	2.6
Stenotrophomonas maltophilia[b]	82.0	5.3
Enterobacteriaceae	**2.0**	**15.8**[c]
Citrobacter diversus/amalonaticus	0	2.6
Enterobacter agglomerans	0	2.6
Enterobacter cloacae	0	2.6
Enterobacter sakazakii	2.0	0
Erwinia nigrifluens	0	2.6
Escherichia coli	0	2.6
Klebsiella oxytoca	0	5.3
Klebsiella pneumoniae subsp. *pneumoniae*	0	7.9
Serratia liquefaciens	0	2.6
Unidentifieid/lost	6.0	0
Total aerobic and facultatively anaerobic gram-negative rods[b]	**84.0**[c]	**26.3**[c]
Cryptococcoideae	**12.0**	**28.9**
Candida albicans	6.0	23.7
Candida guilliermondii	4.0	0
Candida parapsilosis	0	2.6
Candida tropicalis	2.0	2.6
Saccharomycetaceae	**0**	**10.5**
Saccharomyces cerevisiae	0	10.5
Unidentified/lost	2.0	0
Total yeasts[b]	**14.0**	**39.5**

[a] Children group, n = 50; adult group n = 38

[b] Significantly different in prevalence between child and adult groups, Fisher's Exact test, $P < 0.02$.

[c] Total or sub-total value does not add up because some samples contain more than one species of the same family.

Table 2.
Quantity (colony-forming units/ml) of cultivable aerobic and facultatively anaerobic gram-negative rods and yeasts from oral rinse samples[a].

	Child		Adult	
Pseudomonas fluorescens/putida	0		3.7 ± 21.9	(0 – 135)[b]
Pseudomonas paucimobilis	0		3.6 ± 21.9	(0 – 5)
Stenotrophomonas maltophilia	430.1 ± 1573.0	(0 - 1×10^4)	1.5 ± 8.6	(0 – 53)
Citrobacter diversus/amalonaticus	0		0.5 ± 3.2	(0 – 20)
Enterobacter agglomerans	0		3.1 ± 193	(0 – 119)
Enterobacter cloacae	0		0.1 ± 0.8	(0 – 5)
Enterobacter sakazakii	1.7 ± 12.2	(0 – 86)	0	
Escherichia coli	0		72.7 ± 448.4	(0 - 2764)
Erwinia nigrifluens	0		0.1 ± 0.8	(0 – 5)
Klebsiella oxytoca	0		74.1 ± 448.2	(0 – 2764)
Klebsiella pneumoniae subsp. *pneumoniae*	0		3.8 ± 19.5	(0 – 119)
Serratia liquefaciens	0		3.1 ± 19.3	(0 – 119)
Unidentifieid/lost	12.8 ± 72.6	(0 – 498)	0	
Total aerobic and facultatively anaerobic gram-negative rods	**444.7 ± 1570.6**	**(0 - 1×10^4)**	**166.5 ± 621.9**	**(0 – 5528)**
Candida albicans	3.6 ±23.1	(0 – 163)	43.8 ± 130.8	(0 – 676)
Candida guilliermondii	2.1 ± 12.4	(0 – 86)	0	
Candida parapsilosis	0		1.5 ± 9.1	(0 – 56)
Candida tropicalis	9.7 ± 68.3	(0 – 483)	0.4 ± 2.4	(0 – 15)
Saccharomyces cerevisiae	0		3.7 ± 13.0	(0 – 61)
Unidentified/lost	0.5 ± 3.5	(0 – 25)	0	
Total yeast	**15.8 ± 72.3**	**(0 – 483)**	**57.2 ± 137.5**	**(0 – 676)**

[a] Children group, n = 50; adult group n = 38.

[b] mean ±SD and range (in parenthesis).

Table 3.

Distribution of *Stenotrophomonas maltophilia* clonal types among culture positive 11-13-year-old urban Tibetan School Children.

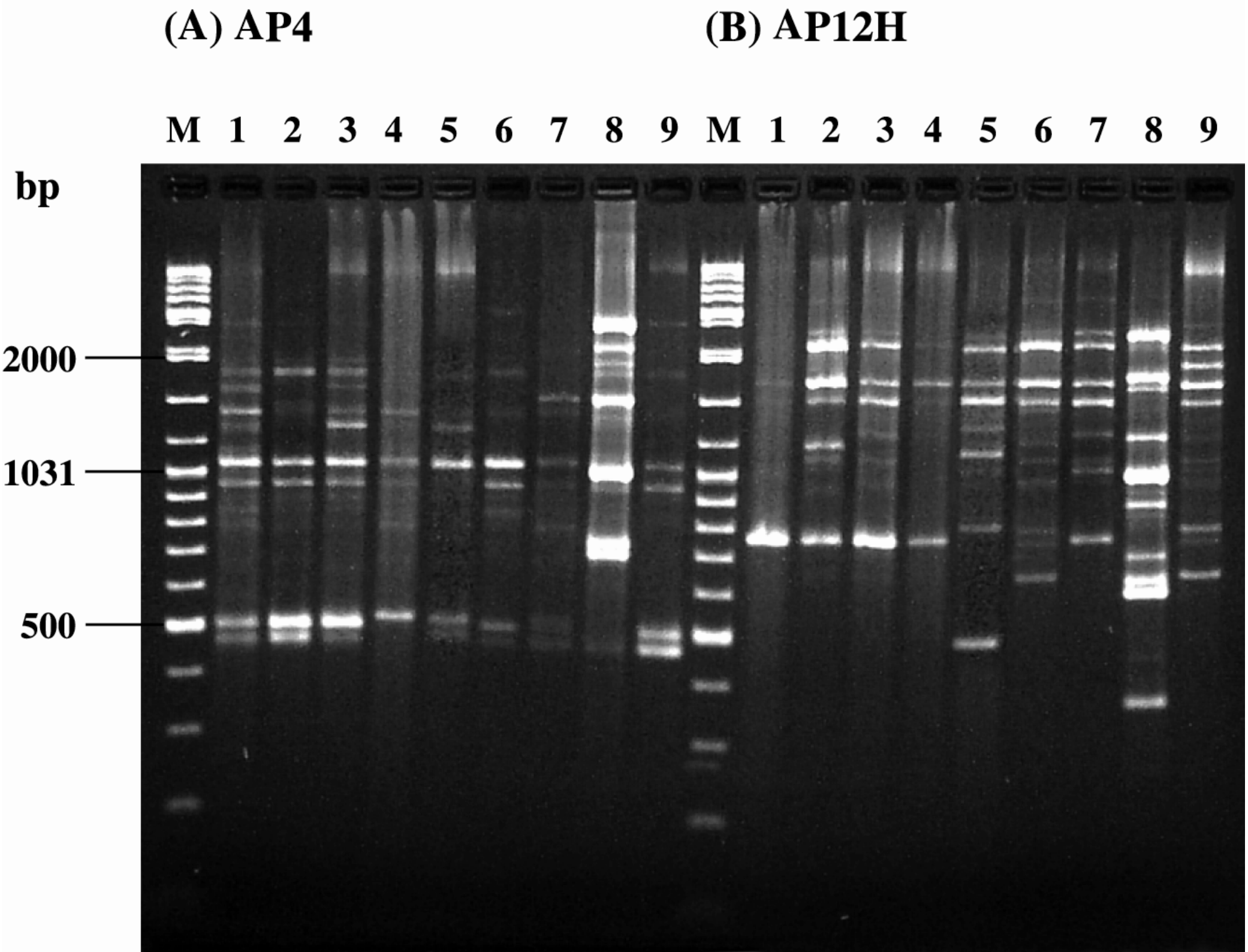
Clonal type	School	
	A	B
AI	1 (2.2)[a]	0 (0)
AII	0 (0)	6 (46.2)[b]
AIII	9 (20.0)	1 (7.7)
AIV	4 (8.8)	2 (15.4)
BI	1 (2.2)	0 (0)
BII	2 (4.4)	0 (0)
BIV	6 (13.3)	1 (7.7)
CV	4 (8.8)	0 (0)
Others[c]	18 (40.0)	1 (7.7)[d]

[a] count and percentage (in parenthesis).

[b] significantly less AII clonal type from isolates recovered from School A, $P < 0.001$, Fisher's Exact test

[c] S_{AB} value > 0.75

[d] Significantly more "others" clonal types from isolates recovered from School A, $P < 0.05$, Fisher's Exact Test.


(A) AP4
(B) AP12H
M 1 2 3 4 5 6 7 8 9 M 1 2 3 4 5 6 7 8 9
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