The genetic population structure of *Mycobacterium bovis* strains isolated from cattle slaughtered at the Yaoundé and Douala abattoirs in Cameroon

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Summary

Bovine tuberculosis is still prevalent and under-evaluated in cattle destined for human consumption in Cameroon. Potential reservoirs for these outbreaks include livestock imported from countries endemic for bovine tuberculosis, such as Nigeria and Chad, and potential residual

reservoirs in local livestock and wildlife. Few studies have been done in Cameroon to genotype the *Mycobacterium tuberculosis* complex (MTC) strains responsible for bovine tuberculosis. The aim of this work is to describe the population structure of MTC strains isolated from cattle, using spoligotyping as the genotyping method.

Out of 218 organs or tissues from cattle with suspected tuberculosis lesions, 90 MTC strains were isolated and underwent molecular typing; among them, 86 strains were identified as *M. bovis* and four strains as *M. tuberculosis*. The *M. tuberculosis* strains belonged to rare *M. tuberculosis* lineages of the U family; among the *M. bovis* strains SB0944 was the most prevalent. Eight new spoligotype patterns were identified, representing 33% (30/90) of all isolates. Among these new spoligotypes, SB1955 was dominant. The spoligotype patterns of 85 *M. bovis* strains lacked spacer 30, a common characteristic of the *M. bovis* lineage African 1, described earlier in Cameroon, Chad, Mali and Nigeria.

This study shows ongoing tuberculosis transmission involving *M. bovis* lineages not previously described as the leading cause of disease. It also shows a possible reverse zoonosis from humans to cattle.

Keywords

Bovine tuberculosis – Cattle – Lineage – *Mycobacterium bovis* – *Mycobacterium tuberculosis* – Spoligotyping.

Introduction

Bovine tuberculosis (TB) is an endemic infectious disease of cattle. It is mainly caused by *Mycobacterium bovis*, which can also cause disease in humans and in a variety of domestic and wild animals (1). *Mycobacterium bovis* is a member of the *M. tuberculosis* complex (MTC), which includes *M. africanum*, *M. microti*, *M. canettii*, *M. pinnipedii* and *M. tuberculosis*. *M. tuberculosis* also causes TB in humans and in domestic and wild animals that are in contact with infected humans (2).

The survey and identification of *M. bovis* is critical for determining the impact of the zoonotic transmission of TB to humans, because it gives impetus to the adoption of public health measures such as pasteurisation of milk, proper cooking of meat, and control of TB in domestic animals (3).

In Cameroon, data on bovine tuberculosis are based mostly on slaughterhouse reports made by veterinarians examining gross macroscopic lesions of TB, and also through the occasional use of tuberculin testing (4). The use of these diagnostic tools has shown that TB is still endemic, with a prevalence ranging from 0.2% to 4.3% for TB macroscopic lesions (5, 6, 7) and 4.67% when using tuberculin testing (8). However, these techniques cannot distinguish among members of the MTC. Moreover, molecular typing studies of the MTC, including spoligotyping, which can provide a rapid means to discriminate members of this complex and which is particularly recommended for rapid analysis of the population structure of *M. bovis* (9), are not routinely applied and are used in very few regions. The only study that has analysed the molecular population structure of *M. bovis* strains in cattle in Cameroon was performed in three regions of the country about 20 years ago (10). It was therefore thought relevant to evaluate the current population structure of MTC strains responsible for bovine TB in Cameroon. Such an analysis would be an important step in the fight against bovine TB because it could increase understanding of the impact of different *M. bovis* genotypes on bovine TB.

Molecular typing of bacterial isolates on the basis of polymorphisms in genomic DNA provides a powerful approach for distinguishing MTC strains and may provide valuable insight into the importance of different hosts and geographical regions in the maintenance and transmission of infection (11, 12). Several methods, including restriction fragment length polymorphism analysis (13, 14), spoligotyping (15) and other polymerase chain reaction (PCR)-based techniques (12), for example those assessing the variability in chromosomal minisatellite regions, have been used to genotype strains of the MTC and for epidemiological studies on human and bovine TB.

Spoligotyping, one of these genotyping techniques, is a simple, rapid and cost-effective method that has been used widely to define predominant clades and to identify a growing number of important clades worldwide (14, 16, 17, 18). It has proven to be useful in genotyping *M. bovis* isolates from cattle, especially those containing few copies of insertion sequence (IS6110) (3, 19). It is recommended as the best technique for analysis of large-scale screening studies of *M. bovis* (9). In Cameroon, very few molecular studies have used spoligotyping, but they have shown that it is a discriminatory and practical tool for molecular typing of *M. bovis* strains isolated from cattle in the country (10).

Materials and methods

Abattoir sampling

The two principal abattoirs chosen for this study were the abattoir of Yaoundé SODEPA (*Société de Développement et d'Exploitation des Productions Animales*) in the Centre region (3°N, 11°E) and that of Douala SODEPA in the Littoral region of Cameroon (4°N, 10°E). The choice of these slaughterhouses was based on the fact that they receive cattle from almost all cattle-breeding regions in Cameroon.

Lesion sampling

Sampling for TB lesions was carried out during routine post-mortem inspection of cattle slaughtered in the two abattoirs. Inspections took place between November 2010 and April 2011, according to the government's legislation regulating veterinary health inspection and notification of contagious animal diseases (20). Approximately 16,316 cattle were slaughtered and inspected for TB during this study. Among them, 9,127 and 7,189 were slaughtered in the Yaoundé and Douala SODEPA abattoirs, respectively. The procedure for sampling of suspected TB lesions was the same as used in previous studies (5).

Processing of samples

The suspected TB lesions were processed by grinding and decontamination with sodium lauryl sulphate (21). Mycobacteria were

cultured using Löwenstein–Jensen (LJ) medium with or without 0.4% pyruvate, and the samples were incubated at 37°C.

Spoligotyping

For amplification of spacers in the Direct Repeat (DR) locus, cell lysates obtained by heat treatment of isolates at 95°C for 20 min were used. Spoligotyping was performed as described by Kamerbeek and colleagues (15).

Data analysis

The degree of similarity between spoligotypes was calculated using the 1-Jaccard index, and the relationships between the spoligotypes were found using the unweighted pair group method of arithmetic averages (UPGMA). Clonal relationships among strain spoligotypes were constructed with the minimum spanning tree (MST), using the Bionumerics software, version 5.10 (Applied Maths NV, Sint-Martens-Latem, Belgium).

Fisher's exact test was used to estimate the association between the breed or region and genotype, using statistical software R version 2.15.3 (www.r-project.org/). Two-sided *P* values of 0.05 or lower were considered statistically significant.

Family assignment

The spoligotype patterns obtained were first submitted to the *M. bovis* international spoligotype database (www.Mbovis.org) to facilitate the comparison of results from different countries. This allowed elucidation of the distribution of strains and the assignment of a unique identifier to spoligotype patterns that had never been previously described. Second, the same spoligotype patterns were submitted to the SITVIT2 database (www.pasteur-guadeloupe.fr:8081/SITVITDemo/) and assigned to families.

Results

Population structure of strains of the *Mycobacterium tuberculosis* complex

To identify the MTC strains, the 90 (41.28%) strains cultured from 218 suspected lesions collected from 218 cattle were spoligotyped. All 90 strains were identified as being of the MTC. Among them, 86 isolates had the specific spoligotype signature of *M. bovis*, meaning that they lacked spacers 3, 9, 16 and 39–43 (Fig. 1), while the remaining four had spoligotype patterns of *M. tuberculosis* (Fig. 1).

The 90 MTC strains were split into 21 different spoligotypes. Among them, nine spoligotypes were new: they had not been described previously, either in the *M. bovis* international designation of spoligotype patterns (www.Mbovis.org) or in the international spoligotype database (SITVIT2). These nine types represented 33% (30/90) of the isolates (Fig. 1). Eight of the nine new spoligotypes were submitted to the *M. bovis* database (www.Mbovis.org) and the numbers assigned were: SB2033, SB2034, SB2035, SB2036, SB1955, SB1956, SB1957 and SB1958. The last of the nine types (CN11), which was representative of the *M. tuberculosis* spoligotype, was identified as belonging to the U family (Fig. 1).

The 86 *M. bovis* strains were regrouped into 19 spoligotypes, all characterised by the lack of spacer 30 except the SB1102 lineage. Spoligotype lineage SB0944 was identified as dominant and accounted for 28% (24/86) of the *M. bovis* strains. The second most prevalent spoligotype (SB1955) accounted for 18.6% (16/86) and was new to the database, while the third (SB0953) accounted for 14% (12/86) of the *M. bovis* strains.

The comparison of *M. tuberculosis* spoligotypes in the international database SITVIT2 permitted identification of all four *M. tuberculosis* strains as belonging to the U family. Among them, one strain belonged to clade ST523, which had not been described in Cameroon previously, in humans or in other animals. The other three strains were new and were characterised by a lack of spacers 15 and 39.

Geographical distribution of strains

The slaughtered cattle examined in this study originated from the main breeding regions of Cameroon, i.e. the Adamawa, the Northern and the Eastern regions (Table I). Spoligotype SB0944 was identified in strains isolated from cattle originating from all three regions, with no statistically significant differences among the regions, while SB1025 and SB0953 were identified in strains isolated from cattle from the North and Adamawa regions. Interestingly, the clades SB1955, SB1956, SB1957, SB1958, SB1459 and SB2033 were exclusively identified in strains isolated from cattle from Adamawa and the clades SB1460, SB0951, SB2034, SB2035, SB2036, SB1439 and SB1099 were identified in strains isolated from cattle originating exclusively from the Northern region.

Distribution of genotypes by cattle breed

Three principal cattle breeds that are bred in Cameroon were identified among the cattle slaughtered in the two abattoirs selected for this study. These cattle breeds were the Mbororo, the Akou and the Goudali (Table I).

Spoligotype SB0944 appeared to be widely distributed among the three breeds of cattle identified in this study, with no statistically significant differences. However, spoligotype SB1955 was only identified in Goudali cattle: all 16 strains belonging to this clade were isolated from this breed. Four spoligotypes (SB1956, SB1957, SB1958 and SB2033) were also specifically identified in this breed. These five spoligotypes had previously not been described but they were all characterised by a lack of the spacers 8, 10 to 13, 15 and 17. Three of the *M. tuberculosis* strains were also identified in the Goudali breed. Strains of spoligotypes SB0951, SB1460, SB2034, SB2035, SB1099 and SB2036 were isolated specifically from Mbororo cattle. However, some *M. bovis* types were identified in two cattle breeds. For example, the spoligotype SB1103 was identified in Mbororo and Akou cattle. SB1418 was identified in both Goudali and Akou cattle, but in small numbers (one isolate from each breed).

Discussion

Characterisation of prevailing MTC lineages focusing on different geographical levels such as continents, countries or regions is important for locating the origin, evolution and transmission dynamics of a particular member of a *M. tuberculosis* clone, which is often difficult to identify by traditional epidemiological investigations alone. As in most of sub-Saharan Africa, bovine TB is prevalent in Cameroon, but to a lesser extent than in neighbouring countries such as Nigeria and Chad (5, 6, 7, 8). In Cameroon, there have been few studies of the population structure of the *M. bovis* strains responsible for bovine TB in slaughtered cattle.

In this study, 90 MTC strains were isolated from lesions found in slaughtered cattle in the main abattoirs of Douala and Yaoundé in Cameroon. The results show that *M. bovis* is still the leading cause of gross macroscopic TB lesions in cattle slaughtered and destined for human consumption in Cameroon (5, 10). From the 90 MTC strains isolated, 21 spoligotypes were identified. Among them, 19 spoligotypes were specific to *M. bovis* and these were all characterised, with the exception of SB1102, by the consistent absence of spacer 30. This trait is characteristic of the strains isolated in Northern Cameroon by Njanpop-Lafourcade and colleagues (10), and in Chad (17), Nigeria (22) and Mali (23). It was also the main spoligotyping characteristic of a clonally related group of *M. bovis* strains named African 1, which was identified by Müller and colleagues (23) in an international analyses of strains isolated in Chad, Cameroon, Nigeria and Mali.

Analysis of the 19 characteristic spoligotypes of *M. bovis* in the international *M. bovis* databases to determine whether or not they had been previously described revealed that four patterns (SB0944, SB1025, SB1099 and SB0951) had been described previously in neighbouring Chad, in a survey of *M. bovis* by Schelling and colleagues (24), and in neighbouring Nigeria, in a survey of *M. bovis* by Cadmus and colleagues (22). The presence of these strains in both Cameroon and the neighbouring countries may be due to direct cattle

trading, because cattle are imported and exported between these countries. In fact, more than 60% of cattle in Cameroon are involved in bovine transhumance (the seasonal movement of people with their livestock) (25).

Three spoligotypes (SB1102, SB1103, SB1418) identified in this study have been previously described only in Chad, while one spoligotype (SB1439) has been previously described only in Nigeria, but all these spoligotypes were identified in small numbers of isolates (one to three). Eleven spoligotypes were specific to Cameroon in that they had never been described elsewhere, particularly in neighbouring Chad and Nigeria. This was intriguing given the trade links between these countries. It could be explained by the fact that imported cattle from neighbouring countries are sent directly for slaughter and are not mixed with the local breeds.

Specific localisation of spoligotypes in the Cameroonian region was also identified in this study. Similar results have been described by Njanpop-Lafourcade and colleagues (10), who analysed Cameroonian *M. bovis* strains isolated during the periods 1989–1990 and 1995–1996. This could be due to specific localisation of cattle breeds in certain regions due to tribal cultures, and also to regional trade limitations (20, 25). This hypothesis is supported by the fact that the spoligotypes specific to Adamawa were identified in strains isolated from the Goudali breed, which is specifically bred in this region (25), while the spoligotype specific to the Northern region was identified in strains isolated from the Mbororo breed, which is more often bred in the far north of the country (25). This was also mentioned by Haddad and colleagues (26), who suggested that the diversity of spoligotypes could be explained by the high diversity of cattle breeds. However, the differences could also be explained by a recent clonal expansion of some of the strains.

An interesting finding in this study is the persistence of spoligotype SB0944, which has remained dominant since 1989, and its wide geographical distribution in Cameroon and in the neighbouring countries (10). This spoligotype was described as dominant by

Njanpop-Lafourcade and colleagues in a study of Cameroonian *M. bovis* strains isolated and conserved since 1989 (10). The same results have been found in France and in some of its neighbouring countries with the characteristic bacillus Calmette–Guérin (BCG)-like SB0120 spoligotype (26), which is also dominant and persistent. Figure 1 shows that the strains harbouring spoligotypes SB0944 and SB0120 had a very high degree of similarity, when considering the method used. Based on this finding, several studies of strains with closely related sequences in the DR locus have concluded that the evolutionary trend of this region of the genome is primarily associated with the loss of single or multiple contiguous spacers (27, 28, 29). As suggested previously (10), it is possible that, following the introduction of French breeds of cattle into Cameroon during the colonial period, the SB0944 spoligotype pattern could have evolved from a French (BCG-like) strain by the loss of spacer 30. Moreover, because all except one *M. bovis* strain isolated in this study lacked the spacer 30, these strains may have evolved from a SB0944 spoligotype by the consecutive loss of contiguous spacers or a group of spacers, or by clonal expansion. The minimum spanning tree analysis (MST) (Fig. 2) showed that such a link could exist between SB0944 and all the other spoligotypes, with a progressive loss of spacers.

Spoligotype SB0944 was identified in all three cattle breeds included in this study. This suggests that the strains presenting this spoligotype may have shared a common source of infection, but it could also reflect the high pathogenicity of the strains harbouring this spoligotype, or an enhanced ability to adapt to many types of geographical and ecological conditions.

Another interesting finding is the identification, selection and adaptation of the second new prevalent spoligotype (SB1955) and other new spoligotypes (SB1956, SB1957, SB1958) in the Goudali breed, which was considered to be more resistant to TB than the Mbororo breed in Cameroon (4). The reason for the selection of these new spoligotypes, especially SB1955, is unknown but it is probably due to the localisation of this breed in the Adamawa region, where it is specifically bred, and the limitation of the circulation of other breeds

in this region by a bill (N°76/420) that was introduced by the Ministry of Livestock, Fisheries and Animal Industries in 1976.

The spoligotyping results also suggest that the *M. bovis* strains isolated in Cameroon since 1989 form a dynamic population. This is supported by the fact that nine new spoligotypes were identified in this study, representing 33% (30/90) of all the strains isolated, in comparison to the findings of Njanpop-Lafourcade and collaborators (10), who genotyped Cameroonian strains isolated in 1989–1990 and 1995–1996. Moreover, only three (SB0944 or C1, SB0951 or C2 and SB0953 or C7) of the ten spoligotypes identified by Njanpop-Lafourcade and colleagues (10) persisted in the same region in this study, 16 years later. Similar results were highlighted by Haddad and colleagues (26) in a survey of French *M. bovis* strains.

Four *M. tuberculosis* strains were isolated in this study. The spoligotypes presented by these strains were identified as belonging to the U family and have not been found earlier in Cameroon, either in humans or in other animals. One of these spoligotypes (ST523) is rare; it has been described in a small number of strains (one to four) from 12 countries, including France, Nigeria and the United States. The other spoligotype (CN11) was novel. The identification of *M. tuberculosis* in cattle is intriguing, but it is known that *M. tuberculosis* can cause TB in such animals (2). Similar results have been reported in neighbouring Nigeria by Cadmus and collaborators (22). Moreover, transmission of *M. tuberculosis* from humans to cattle has been reported (30). The results of this study may indicate human-to-cattle transmission of TB in Cameroon, because there is close contact between humans and livestock.

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Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Ethical considerations

Institutional permission to conduct the study was obtained from the Ministry of Livestock, Fisheries and Animal Industry, Cameroon.

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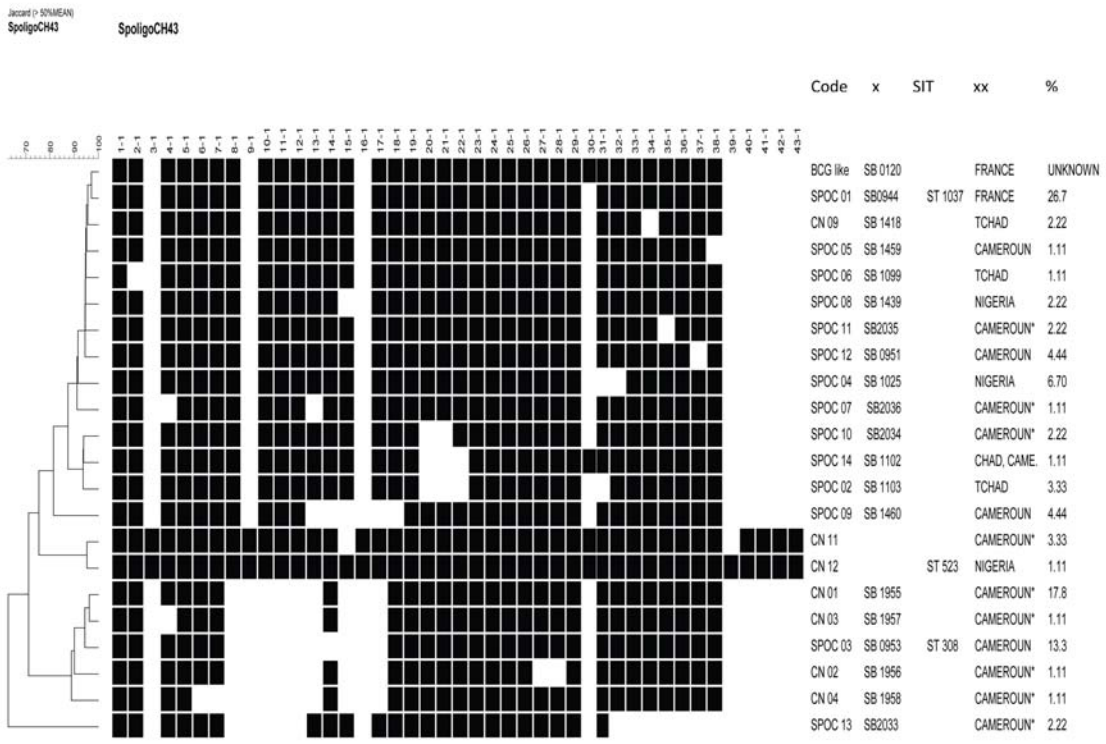


CN: Spoligotype isolated in Cameroon Central region; the number represents the series number of the spoligotype

SIT: Shared International Type

SPOC: Spoligotype isolated in Cameroon; the number represents the series number of the spoligotype

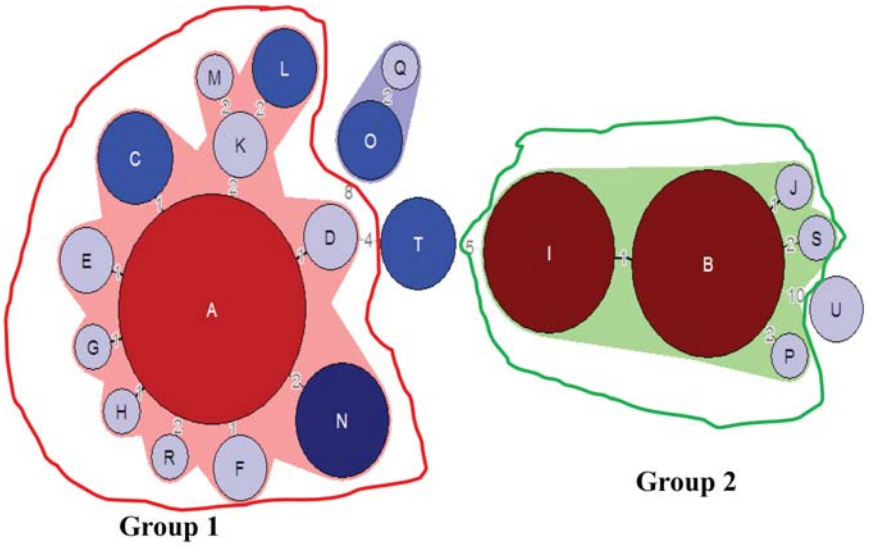
X: SB number

XX: First country of isolation

Fig. 1

Dendrogram showing the relationships among the 21 spoligotypes of 90 strains of the *Mycobacterium tuberculosis* complex isolated from cattle in Cameroon

The degree of similarity between spoligotypes was calculated using the 1-Jaccard index and the relationships between the spoligotypes were found using UPGMA (unweighted pair group method of arithmetic averages)



Each spoligotype is represented by a letter:

A: SB0944; B: SB1955; C: SB0951; D: SB1439; E: SB2035; F: SB1418; G: SB1099; H: SB14559; I: SB0953; J: SB1957; K: SB2034; L: SB1102; M: SB1103; N: SB1025; O: CN11; P: SB1958; Q: ST523; R: SB2036; S: SB1956; T: SB1460; U: SB2033

Fig. 2

Minimum spanning tree diagram showing expansion of or between the 21 spoligotypes of 90 strains of the *Mycobacterium tuberculosis* complex isolated from infected cattle in Cameroon

The size of each circle is proportional to the number of isolates belonging to that spoligotype

Table I

Distribution of spoligotypes in the main cattle-breeding regions of Cameroon, according to breed and region

		Goudali breed			Akou breed			Mbororo breed			Unknown breed	Total
SB number	SIT	Adamawa	Northern	Eastern	Adamawa	Northern	Eastern	Adamawa	Northern	Eastern	Unknown	
SB0944	1,037	8	0	0	1	3	0	1	6	4	1	24
SB1103		0	0	0	0	0	1	0	2	0	0	3
SB0953	308	3	0	0	0	0	0	0	4	0	5	12
SB1025		2	0	0	0	0	0	0	4	0	0	6
SB1459		1	0	0	0	0	0	0	0	0	0	1
SB1099		0	0	0	0	0	0	0	1	0	0	1
SB2036		0	0	0	0	0	0	0	1	0	0	1
SB1439		0	0	0	0	0	0	0	0	0	2	2
SB1460		0	0	0	0	0	0	0	4	0	0	4
SB2034		0	0	0	0	0	0	0	2	0	0	2
SB2035		0	0	0	0	0	0	0	2	0	0	2
SB0951		0	0	0	0	0	0	0	4	0	0	4
SB2033		2	0	0	0	0	0	0	0	0	0	2
SB1102		0	0	0	0	0	0	0	0	0	1	1
SB1955		16	0	0	0	0	0	0	0	0	0	16
SB1956		1	0	0	0	0	0	0	0	0	0	1
SB1957		1	0	0	0	0	0	0	0	0	0	1
SB1958		1	0	0	0	0	0	0	0	0	0	1

		2	0	0	0	0	1	0	0	0	0	3
	523	1	0	0	0	0	0	0	0	0	0	1
SB1418		1	0	0	1	0	0	0	0	0	0	2
	TOTAL	39	0	0	2	3	2	1	30	4	9	90

SIT: Shared International Type