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Multiplex PCR to determine *Streptococcus pneumoniae* serotypes causing otitis media in the Republic of Ireland with further characterisation of antimicrobial susceptibilities and genotypes

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ABSTRACT

Purpose: To determine the serotypes, genotypes and antimicrobial susceptibilities of *Streptococcus pneumoniae* causing otitis media (OM) in children in Dublin, Ireland.

Methods: *S. pneumoniae* isolates (n = 28) from spontaneously discharging OM were studied. Serotyping was performed using a previously undescribed multiplex PCR scheme in combination with serological methods. Multilocus sequence typing (MLST) was performed using standard procedures. Antimicrobial susceptibility testing was performed using the Etest method.

Results: Fourteen different *S. pneumoniae* serotypes were identified. The five most common serotypes were 3, 19F, 19A, 14 and 6A which accounted for 68% of all infections. The 7-valent pneumococcal conjugate vaccine (PCV7), 10-valent pneumococcal conjugate vaccine (PHiD-CV) and 13-valent pneumococcal conjugate vaccine (PCV13) provided potential coverage of 43%, 46% and 86%, respectively.

Reduced susceptibility to penicillin was evident for 25% of isolates and associated with serotypes 14, 19A, 19F and 9V. A total of 21 different sequence types (STs) were identified. ~~, 54% of which were representatives of Pneumococcal Molecular Epidemiology Network (PMEN) clones or their~~ or their variants represented 54% (15/28) of all isolates.

~~and 40% of these demonstrated reduced susceptibility to at least one antimicrobial agent.~~

Conclusions: Continued monitoring and characterisation of *S. pneumoniae* causing OM in Ireland is warranted in order to guide future vaccine and treatment policies.

KEYWORDS

Streptococcus pneumoniae; otitis media; serotype; PCR

INTRODUCTION

Streptococcus pneumoniae is one of the leading causes of otitis media (OM) in children and is responsible for approximately 38% of all cases worldwide [1]. The majority (83%) of children less than 3 years of age experience at least one episode of OM [2]. The cost of outpatient visits and treatments due to OM is considerably high, with an estimated expense of \$2-5 billion per annum in the United States (US). The seven-valent pneumococcal conjugate vaccine (PCV7; Prevenar 7™) was or continues to be routinely administered to young children in a number of countries. An overall reduction of 6% and 8.9% in clinical OM incidence was demonstrated in Finnish and US infants following immunisation with PCV7 [3]. Vaccine efficacy of 10-50% was evident against recurrent OM or for the prevention of tympanostomy tube placement. A decline of 42.7% and 41.9% in OM associated ambulatory visits and antibiotic prescriptions since vaccine introduction in the US, respectively, was demonstrated by Zhou et al. [2].

PCV7 was introduced to the infant immunisation programme in Ireland in late 2008. In order to determine the effects and impact of PCV7 in Ireland and indeed elsewhere, it is important to have an understanding and knowledge of *S. pneumoniae* serotypes causing infection. To our knowledge, no data associated with the epidemiology of *S. pneumoniae* causing OM in Ireland has been documented.

Accurate *S. pneumoniae* identification is essential for diagnosis and for optimal treatment. Reliable typing systems are important to detect changes in the dominant strains over time. Our group previously developed a multiplex PCR assay to identify the seven serotypes contained in PCV7 [4]. Here we describe a new and improved multiplex PCR scheme for use on lysed single colony extracts that confirms *S. pneumoniae* identification

and detects an expanded number of serotypes. In addition, we describe the serotypes, genotypes and antimicrobial susceptibilities of *S. pneumoniae* causing OM in children in Ireland.

METHODS

Study population.

S. pneumoniae isolated from swabs of spontaneous otological drainage in children with OM, received by the routine microbiology laboratory of the Children's University Hospital, Temple St., Dublin, an acute and tertiary referral paediatric hospital, were included in the study. Between January 2007 and December 2008, 28 *S. pneumoniae* isolates were identified by traditional microbiological methods following culture on Columbia blood agar plates. PCV7 was introduced to the immunization program in Ireland in September 2008, thus, three isolates were recovered after PCV7 introduction.

Bacterial isolates control collection. A control collection (n=144) of *S. pneumoniae* isolates, whose serotype had been previously determined [5], were retrieved from storage in 1:3 glycerol (nutrient broth containing 0.5% glucose and 10% glycerol)-horse serum storage medium at -70°C. Of these, 132/144 (92%) isolates had remained viable and were available for reserotyping.~~identified using standard microbiological methods.~~ An additional control collection of 51 Statens Serum Institute (SSI) (Copenhagen, Denmark) *S. pneumoniae* strains were also utilised to validate the PCR assays. To ensure accurate *S. pneumoniae* identification, other organisms used to test the specificity of the assay included well characterised isolates of 21 bacterial species including *S. agalactiae*, *S. pyogenes*, *Neisseria meningitidis* and *Haemophilus influenzae*. Additional clinical isolates of viridans group streptococci (n=20) were also assessed to ensure no cross reactivity occurred.

DNA preparation. A single colony from a fresh overnight culture grown on Columbia blood (5% sheep blood) agar plates was immersed in 20µl Microlysis™ Plus (Microzone

Ltd., Sussex, UK) and processed according to manufacturer's guidelines. PCR was then performed on the lysed single colony extracts.

Multiplex PCR assembly. All available relevant sequences were aligned using ClustalX, and the appropriate primers (9V-F, 3-R, *lytA*-F and *lytA*-R) determined using Primer Express[®] (Applied Biosystems, Warrington, UK) software. The *lytA* primers were designed to target conserved nucleotides in typical *lytA* alleles, characteristic of pneumococci not present in *lytA* alleles of mitis group streptococci [6-8]. The primer sequences and amplicon size of products were as previously described, with the exception of final primer concentrations in each reaction mixture; serotype 4 (0.75 μ M) and 14 (0.5 μ M) [9], 6 (0.6 μ M) [10], 18 (0.75 μ M), 19F (0.6 μ M), 23F (0.75 μ M), 1 (0.4 μ M), 5 (0.48 μ M), 7F (0.64 μ M) and *cpsA* (0.08 μ M) [11]. A serotype 9V forward primer was designed (5'-agttcaccttgtaacagttatcccata-3') (0.6 μ M) and used in combination with a previously described 9V reverse primer [11] (0.6 μ M), which yielded a product of 107bp. A new serotype 3 reverse primer was also designed (5'-ttctgccaccttagttgct-3') (0.4 μ M) and used in combination with a previously described forward primer [10] (0.4 μ M), which yielded a product of 470bp. The newly designed *lytA* forward primer (5'-caactaaagaagagttcatga-3') (0.1 μ M) and *lytA* reverse primer (5'-ctcgtgcgttttaattccagc-3') (0.1 μ M) yielded a product of 125bp.

The first multiplex PCR reaction was designed to screen for serotypes contained in PCV7 with the use of *cpsA* primers as an internal positive control (IPC) [9,11] (Figure 1). The second multiplex PCR reaction was designed to confirm *S. pneumoniae* identification (*lytA*) and screen for serotypes 1, 3, 5 and 7F, with *cpsA* as an IPC.

PCR reaction. Each 25µl PCR reaction mixture contained the following: 1x reaction buffer (Tris-Cl, KCl, (NH₄)₂SO₄, 1.5mM MgCl₂; pH 8.7; Qiagen), 1mM MgCl₂ (Qiagen), 1x Q-solution (Qiagen), 200µM of each deoxynucleoside triphosphate (Promega Inc.), 2U Hot Star Taq DNA polymerase (Qiagen) and 1µl of lysed Microlysis[®] Plus colony extract as template. Thermocycling was performed on a Peltier Thermal Cycler 200 DNA engine (MJ Research, Massachusetts, USA) using the following conditions: 95°C for 15min, followed by 35 cycles at 95 °C for 40s, 55 °C for 45s, 65 °C for 150s and a final extension step at 65 °C for 1min. PCR products (10µl) were analysed using 3% (w/v) agarose gel electrophoresis with 5µg/ml ethidium bromide and visualised under UV transillumination. PCR product sizes were compared with the molecular size standard (100bp ladder; New England Biolabs, Beverley, MA).

Serotyping.

Isolates were serotyped using a combination of the multiplex PCR scheme described above, and/or for those isolates for which the serotype was not determined using the above method, the serotype was determined by serological methods [12] using antisera obtained from the Statens Serum Institute (Copenhagen, Denmark).

DNA isolation and multilocus sequence typing (MLST).

DNA was purified from fresh overnight cultures grown on Columbia blood agar using the Genra[®] Puregene[®] Yeast/Bact. kit (Qiagen) according to manufacturer's guidelines. MLST was performed using the standard procedure to amplify the seven house-keeping genes as previously described [13]. Allele numbers and sequence types (STs) were assigned and eBURST analysis performed using www.mlst.net. Genetically related clones were either single locus variants (SLVs) or double locus variants (DLVs) of a

specific clone. STs were compared with those of the PMEN clones available at <http://www.sph.emory.edu/PMEN/>.

Antimicrobial susceptibility.

Isolates were tested for susceptibility to penicillin, cefotaxime, erythromycin, clindamycin and tigecycline using the Etest (AB bioMérieux, Solna, Sweden) method and results were interpreted following CLSI guidelines [14]. Susceptibility to penicillin was defined as follows: susceptible, MIC $\leq 0.06\mu\text{g/ml}$; intermediate, $0.12\text{-}1\mu\text{g/ml}$; and high-level resistant, $\geq 2\mu\text{g/ml}$; corresponding to the new oral penicillin breakpoints. Isolates with reduced susceptibility to three antibiotics or more were considered multidrug resistant (MDR).

RESULTS

Multiplex PCR validation. The multiplex PCR reactions correctly identified 73.5% (97/132) of all relevant serotypes targeted in the control collection of isolates (Table 1).

Three discrepancies did occur. However, repeat assessment confirmed the PCR result as accurate. The *cpsA* primers (internal positive control) were consistently positive for all isolates and the 90 pneumococcal serotypes tested (Figure 1). Exceptions included lack of reactivity with isolates harbouring the type 25A, 25F and 38 capsule, as previously observed [15]. The *lytA* primers consistently and specifically amplified all pneumococci tested, with no cross reactivity with other organisms identified.

Patient characteristics.

Twenty eight cases of OM caused by *S. pneumoniae* were identified in children, age range 23 days to nine years. The majority (68%) of infections occurred in children <3 years. The male: female ratio was 1.5:1.

Serotype distribution.

A total of 14 different *S. pneumoniae* serotypes were identified. The most common serotypes were 3 (n=5) and 19F (n=5), followed by 19A (n=3), 14 (n=3) and 6A (n=3).

These five serotypes together accounted for 68% of infections overall. Three isolates caused infection after PCV7 introduction, one expressed vaccine serotype 14, another expressed vaccine serotype 9V, and the third isolate expressed serotype 6A. The potential coverage provided by PCV7 ~~withi~~ all age groups included was 43%, PHiD-CV (Synflorix™, GlaxoSmithKline plc., UK) was 46% and PCV13 (Prevenar 13, Pfizer Inc., USA) was 86%. For those less than two years of age the potential coverage provided by both PCV7 and PHiD-CV was 53%, increasing to 87% for PCV13.

Sequence Types and Antimicrobial susceptibility.

Reduced susceptibility to penicillin was observed for 25% of the isolates, 86% of which occurred in children <3 years. One isolate (serotype 14) demonstrated high level penicillin resistance and caused an infection in a one year old boy. Four serotypes were associated with reduced susceptibility to penicillin, the most common being serotype 14 (n=3), followed by 19A (n=2), 19F (n=1) and 9V (n=1). Intermediate resistance to cefotaxime was observed for 3.6% of isolates. Resistance to erythromycin and clindamycin was observed for 18% and 11% of isolates, respectively. All isolates were sensitive to tigecycline. MDR was evident for 11% of isolates and all were members of serogroup 19.

Twenty-one STs were identified in total (Table 2). The predominant clone associated with serotype 3 was ST180 (n=3) and the genetically unrelated serotype 3 ST458 (n=1) was also identified. A high level of variation was observed among the clones associated with serotype 19F (ST420, ST425, ST73, ST87 and ST3508), all of which were considered unrelated to each other following eBURST analysis (data not shown). ST87 demonstrated intermediate resistance to penicillin and a very high level of resistance to the macrolide class of antibiotics, namely, erythromycin and also clindamycin ~~(Table 3)~~. All other clones expressing the serotype 19F capsule were sensitive to all antimicrobials tested. The two ST276 (serotype 19A) isolates were MDR. Each of the two ST15 isolates expressing serotype 14 capsules displayed similar antibiograms to each other i.e. intermediate resistance to penicillin and high level resistance to erythromycin ~~(Table 3)~~. High level penicillin resistance was evident for a ST156 isolate expressing a serotype 14 capsule, while another representative of the same

clone expressing a serotype 9V capsule, demonstrated intermediate resistance to penicillin. Two STs were associated with serotype 6A, ST65 (n=2) and ST2611 (n=1), each from a different clonal lineage and susceptible to all antimicrobials tested.

PMEN clones.

Over half (54%) of the isolates in this study were representatives of PMEN clones or their variants while 40% of these demonstrated reduced susceptibility to at least one antimicrobial agent (Table 2; ~~Table 3~~). The most common PMEN clone was Netherlands³-31 (n=4) followed by Spain^{9V}-3, a SLV of Denmark¹⁴-32 and a SLV of England¹⁴-9 with two representative isolates each. Other clones included Sweden⁴-38, a SLV of Tennessee^{23F}-4, a SLV of Tennessee¹⁴-18, and a SLV and DLV of Netherlands^{15B}-37.

DISCUSSION.

This is the first report of the serotype distribution of *S. pneumoniae* causing otitis media in children in Ireland. In order to determine the serotypes associated with these infections, a combination of multiplex PCR and traditional serological methods was employed. During the multiplex PCR validation process using control collections of isolates, no cross reactivity with organisms other than *S. pneumoniae* was identified and the serogroup/serotype of all *S. pneumoniae* isolates targeted by the scheme was accurately determined, although three exceptions were initially identified. Nonetheless, further analysis confirmed the PCR result as accurate.

~~For the control collection of isolates, associated with invasive infections in Ireland and whose serotype was previously determined by the Quellung reaction [5], 74% (97/132) of the serogroups/serotypes were identified using the multiplex PCR scheme.~~
Due to ~~For this reason and also~~ the less laborious nature of the PCR method, in addition to the absence of a DNA purification step, it was considered feasible and more efficient to employ this method in combination with serological methods for serotype determination of isolates in our laboratory setting. A limitation of this PCR method is its inability to differentiate between the commonly occurring serotype 6A, 6B and the more recently described 6C [16]. In addition, this method does not identify serotype 19A, which is a frequently occurring serotype among OM isolates in this study. For these and the other serotypes not targeted with the multiplex PCR assays, confirmation to serotype level using traditional serological methods and/or PCR in the case of 6C, is required.

~~The WHO estimates that complications of OM result in approximately 51000 deaths in children younger than five years of age every year. This infection is also one of~~

~~the most common reasons for sick children to visit their general practitioner/ physician and a major reason for antibiotic prescriptions, especially in the case of recurrent OM, whereby 40% will have six or more recurrences by seven years of age [17].~~ Efforts are being made in a number of countries to reduce antibiotic consumption, as levels of antimicrobial resistance are developing more rapidly than the development of new antimicrobials. However, use of a pneumococcal conjugate vaccine reduced otitis associated antimicrobial consumption by 20% [178]. Of the four serotypes associated with reduced susceptibility to penicillin in this study, three of these are contained in PCV7 and PHiD-CV, while all are contained in PCV13. Reduced susceptibility to penicillin (25% of isolates) was more common than resistance to erythromycin (18%) and clindamycin (11%). This level of penicillin resistance is half that observed (50%) for OM isolates in Spain in the 1990's, where OM clinical failures were associated with 90% penicillin resistance [189]. Conversely, penicillin resistance in Ireland is considerably higher than the 1.5% level observed for *S. pneumoniae* isolates in the Netherlands [1920], a country with one of the lowest rates of antimicrobial prescribing in the world [201].

In the present study, the top five ranked serotypes were 3, 19F, 19A, 14 and 6A, which are also the most common causes of OM worldwide [212]. The potential coverage provided by PCV7 and PHiD-CV was similar at 43% and 46%, respectively. This increased to 86% for PCV13. In the US since PCV7 introduction a serotype 3 clone, namely, Netherlands³-31(ST180) has become more prevalent and is emerging as a common vaccine replacement serotype causing disease [1, 212, 223]. The present study indicates that this particular serotype 3 clone, susceptible to all antibiotics tested, was most commonly associated with OM in Dublin prior to PCV7 vaccine introduction. This

finding is similar to reports from Costa Rica, Israel and Lithuania demonstrating serotype 3 as a common cause of non-invasive infections [223]. ~~Unlike the majority of *S. pneumoniae* serotypes, serotype 3 isolates have a mucoid appearance on colonial morphology.~~ The generation of vaccine efficacy against serotype 3 OM was not demonstrated in a study involving a candidate 11-valent pneumococcal conjugate vaccine [23]. This may have been due to the atypical immune response generated or the high abundance of capsular polysaccharide expression against which antibodies may be less effective [234]. Nonetheless, the recently licensed PCV13 does contain serotype 3.

The serotype 19A strains came from two different clonal lineages. ST276 (serotype 19A), which is a SLV of Denmark¹⁴-32 with a similar antibiogram to the clone that existed prior to PCV7 introduction in France, was also identified. ST276 has undergone clonal expansion in France to represent the predominant 19A replacement clone since PCV7 introduction. A number of OM treatment failures have been associated with serotype 19A [245]. Hence, close monitoring is necessary to determine whether or not the same level of expansion of this serotype occurs in Ireland [256]. The extensively characterised Spain^{9V}-3 (ST156) clone was present expressing a serotype 14 and 9V capsule. Spain^{9V}-3 is a widely disseminated clone commonly associated with penicillin resistance. It seems to have emerged in Spain in the 1980's and has established itself as a common cause of infections in a number of countries such as France, Denmark, Germany and the Netherlands [267-301]. The two serotype 6A clones, ST65 and ST2611, were also from diverse lineages and unlike the multidrug-resistant 6A clones in OM that are emerging in Israel [312], neither clone displayed resistance to the antimicrobials tested.

Overall, the phenotype and genotype of *S. pneumoniae* isolates causing OM in Dublin is similar to that observed in a number of other countries. However, a limitation of the study is the small number of isolates available for characterisation over a relatively short period confined to one region in Ireland. For this reason results should be interpreted with some degree of caution. However, this study has provided important baseline information, for the most part, prior to PCV7 introduction, when serotype replacement due to vaccine introduction is unlikely to have occurred. It is interesting that three of the most common serotypes causing OM are not contained in PCV7, although cross-protection against serotype 6A may be provided, as evidenced by the decline of this serotype since PCV7 introduction in the US [323]. However, serotype 3, 6A and 19A are contained in PCV13, so infections caused by these serotypes are potentially vaccine preventable.

In conclusion, this study reinforces the importance of continued monitoring of the epidemiology of *S. pneumoniae* causing OM in Ireland, especially in the post-PCV7 vaccine era, in order to determine if serotype replacement occurs and to monitor antibiotic resistance. Such monitoring will guide future vaccine and treatment policies.

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Figure legend

Fig. 1 Multiplex PCR products following 3% (w/v) agarose gel electrophoresis. Lane M: Molecular marker (100bp) Lane 1-7: Multiplex PCR reaction 1 PCR products representing serotype 4, 6, 9V, 14, 18, 19F and 23F, respectively. A band representative of *cpsA* (160bp) is evident for each sample. Lane 8-11: Multiplex PCR reaction 2 PCR products representing serotype 1, 3, 5 and 7F, respectively. A band representative of *cpsA* (160bp) is evident for each sample, in addition to *lytA* amplification confirming typical pneumococcal identification.

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Table 1. Validation results of Multiplex PCRs.

<u>Serotype*</u>	<u>Quellung</u>	<u>Multiplex PCR</u>	<u>Multiplex PCR</u>	
	<u>Number</u>	<u>Number</u>	<u><i>cpsA</i></u>	<u><i>lytA</i></u>
<u>14</u>	<u>28</u>	<u>29^a</u>	<u>±</u>	<u>±</u>
<u>9V/(9A)</u>	<u>21</u>	<u>19^a</u>	<u>±</u>	<u>±</u>
<u>4</u>	<u>12</u>	<u>13^a</u>	<u>±</u>	<u>±</u>
<u>23F</u>	<u>4</u>	<u>4</u>	<u>±</u>	<u>±</u>
<u>19F</u>	<u>4</u>	<u>3^a</u>	<u>±</u>	<u>±</u>
<u>6A/6B</u>	<u>4</u>	<u>4</u>	<u>±</u>	<u>±</u>
<u>18C/(18A,18B,18F)</u>	<u>3</u>	<u>3</u>	<u>±</u>	<u>±</u>
<u>7F/(7A)</u>	<u>3</u>	<u>3</u>	<u>±</u>	<u>±</u>
<u>3</u>	<u>9</u>	<u>9</u>	<u>±</u>	<u>±</u>
<u>1</u>	<u>4</u>	<u>5^a</u>	<u>±</u>	<u>±</u>
<u>5</u>	<u>5</u>	<u>5</u>	<u>±</u>	<u>±</u>

*Rarely occurring serotypes not resolved by PCR method are indicated in parentheses.

^aRepeat serology confirmed serotype 14, 4 and 1 PCR results for isolates initially identified as 9V, 19F and 9V.

Table 2. Properties and STs of *S. pneumoniae* isolates causing OM in Ireland

<u>ST</u>	<u>Allele number</u>							<u>Serotype(s)</u>	<u>Total</u>	<u>PMEN clone</u>
	<u>aroE</u>	<u>gdh</u>	<u>gki</u>	<u>recP</u>	<u>spi</u>	<u>xpt</u>	<u>ddl</u>			
<u>180</u>	<u>7</u>	<u>15</u>	<u>2</u>	<u>10</u>	<u>6</u>	<u>1</u>	<u>22</u>	<u>3</u>	<u>4</u>	<u>Netherlands³-31</u>
<u>458</u>	<u>2</u>	<u>32</u>	<u>9</u>	<u>47</u>	<u>6</u>	<u>21</u>	<u>17</u>	<u>3</u>	<u>1</u>	
<u>73</u>	<u>2</u>	<u>13</u>	<u>2</u>	<u>15</u>	<u>6</u>	<u>1</u>	<u>1</u>	<u>19F</u>	<u>1</u>	
<u>87</u>	<u>5</u>	<u>5</u>	<u>7</u>	<u>7</u>	<u>8</u>	<u>5</u>	<u>4</u>	<u>19F</u>	<u>1</u>	
<u>420</u>	<u>15</u>	<u>38</u>	<u>19</u>	<u>5</u>	<u>36</u>	<u>20</u>	<u>6</u>	<u>19F</u>	<u>1</u>	
<u>425</u>	<u>1</u>	<u>5</u>	<u>1</u>	<u>5</u>	<u>1</u>	<u>1</u>	<u>8</u>	<u>19F</u>	<u>1</u>	
<u>3508</u>	<u>47</u>	<u>5</u>	<u>15</u>	<u>5</u>	<u>15</u>	<u>20</u>	<u>8</u>	<u>19F</u>	<u>1</u>	
<u>66</u>	<u>2</u>	<u>8</u>	<u>2</u>	<u>4</u>	<u>6</u>	<u>1</u>	<u>1</u>	<u>9N</u>	<u>1</u>	<u>SLV</u>
										<u>Tennessee¹⁴-18</u>
<u>276</u>	<u>2</u>	<u>19</u>	<u>2</u>	<u>17</u>	<u>6</u>	<u>22</u>	<u>14</u>	<u>19A</u>	<u>2</u>	<u>SLV Denmark¹⁴-</u>
										<u>32</u>
<u>2081</u>	<u>8</u>	<u>5</u>	<u>14</u>	<u>4</u>	<u>17</u>	<u>1</u>	<u>14</u>	<u>19A</u>	<u>1</u>	<u>DLV</u>
										<u>Netherlands^{15B}-</u>
										<u>37</u>
<u>15</u>	<u>1</u>	<u>5</u>	<u>4</u>	<u>5</u>	<u>5</u>	<u>3</u>	<u>8</u>	<u>14</u>	<u>2</u>	<u>SLV England¹⁴-</u>
										<u>9</u>
<u>156</u>	<u>7</u>	<u>11</u>	<u>10</u>	<u>1</u>	<u>6</u>	<u>8</u>	<u>1</u>	<u>14, 9V</u>	<u>2</u>	<u>Spain^{9V}-3</u>
<u>65</u>	<u>2</u>	<u>7</u>	<u>4</u>	<u>10</u>	<u>10</u>	<u>1</u>	<u>27</u>	<u>6A</u>	<u>2</u>	

<u>2611</u>	<u>1</u>	<u>5</u>	<u>6</u>	<u>12</u>	<u>13</u>	<u>20</u>	<u>6</u>	<u>6A</u>	<u>1</u>	
<u>205</u>	<u>10</u>	<u>5</u>	<u>4</u>	<u>5</u>	<u>13</u>	<u>10</u>	<u>18</u>	<u>4</u>	<u>1</u>	<u>Sweden⁴-38</u>
<u>201</u>	<u>8</u>	<u>13</u>	<u>14</u>	<u>4</u>	<u>9</u>	<u>4</u>	<u>14</u>	<u>15C</u>	<u>1</u>	<u>SLV</u>
										<u>Netherlands^{15B}-</u>
										<u>37</u>
<u>439</u>	<u>1</u>	<u>8</u>	<u>9</u>	<u>2</u>	<u>6</u>	<u>4</u>	<u>6</u>	<u>23B</u>	<u>1</u>	<u>SLV</u>
										<u>Tennessee^{23F}-4</u>
<u>36</u>	<u>1</u>	<u>8</u>	<u>4</u>	<u>1</u>	<u>1</u>	<u>4</u>	<u>6</u>	<u>23F</u>	<u>1</u>	
<u>3729</u>	<u>42</u>	<u>35</u>	<u>29</u>	<u>36</u>	<u>9</u>	<u>264</u>	<u>18</u>	<u>18C</u>	<u>1</u>	
<u>191</u>	<u>8</u>	<u>9</u>	<u>2</u>	<u>1</u>	<u>6</u>	<u>1</u>	<u>17</u>	<u>7F</u>	<u>1</u>	
<u>393</u>	<u>10</u>	<u>43</u>	<u>41</u>	<u>18</u>	<u>13</u>	<u>20</u>	<u>6</u>	<u>38</u>	<u>1</u>	

Fig.1

