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MAJOR ARTICLE

Ices*p1109*, a novel hybrid Integrative Conjugative Element of macrolide-resistant *Streptococcus pyogenes* serotype M77 collected between 2003 and 2017 in Poland

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Objectives: Genetic characterization of the antibiotic resistance determinants and associated mobile genetic elements (MGEs) among *Streptococcus pyogenes* [Group A streptococci (GAS)] clinical isolates of an M77 serotype collected in Poland between 2003 and 2017.

Methods: The genomes of 136 M77 GAS isolates were sequenced using Illumina, and selected with long-read approach (Oxford Nanopore). Whole genome sequences were analyzed to determine the presence of macrolide resistance determinants, and their genetic context.

Results: The strains used in the study were collected in the two multicenter surveys from in- and outpatients. Sequencing data analysis revealed that all strains carried the *tet*(O) gene (100%, N=136). They were classified as a single sequence type ST63. For erythromycin resistance, the unique determinant was *erm*(TR) detected in 76.5% (N=104) isolates. A single appearance of

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tet(M) and *erm*(B) on Tn*3872* was noticed. The *mef*A, *mef*E, and *msr*(D) genes were detected in neither of the genomes. This correlated with the detected strain phenotypes – 11 exhibited $cMLS_B$, 93 – $iMLS_B$, and no M phenotype.

The *erm*(TR) gene was predominantly (N=74) found within a novel hybrid Integrative Conjugative Element composed of the ICE*Sp1108*-like sequence and ICE*Sp2906* variant which was then named ICE*Sp1109*. However, in strains isolated before 2008, *erm*(TR) was located within ICE*Sp2905* (N=27). The *erm*(TR) gene was detected within stand-alone ICE*Sp1108*-like sequences in 3 strains.

Conclusions: Based on phylogenetic analysis results the clonal dissemination of the macrolide-resistant *S. pyogenes* M77/ST63 strain with hybrid ICE*Sp1109* was observed between 2008 and 2017. ICE*Sp1109* is the novel hybrid ICE in Gram-positive bacteria.

Keywords: *Streptococcus pyogenes*; macrolide resistance; *erm*(TR); M77/ST63; Integrative Conjugative Element; genome sequencing; hybrid ICE; clonal dissemination

INTRODUCTION

Streptococcus pyogenes (Group A Streptococcus, GAS) is a major human pathogen that causes a wide range of infections from mild, such as pharyngitis or impetigo to severe – invasive ones, such as septicemia, necrotizing fasciitis, and streptococcal toxic shock syndrome (for review [1]). Although all GAS strains remain sensitive to β-lactams, and this class of antibiotics is a drug of choice to treat streptococcal infections, macrolides are recommended for patients allergic to β-lactams. Moreover, macrolide combined with β-lactams is preferred in treating severe or complicated *S. pyogenes* infections [2].

Resistance to macrolides in *S. pyogenes* primarily involves target site modification and depends on the presence of the *erm* genes, which code for a methylase of the ribosomal RNA (23S rRNA). The resulting modification of the ribosome prevents the binding of macrolide antibiotics. The *erm* genes confer resistance not only to macrolides, but also to lincosamides, and streptogramin B, therefore the phenotype presented by the bacteria carrying *erm* genes is called MLS_B (from macrolides, lincosamides, streptogramin B). The expression of the *erm* genes is predominantly inducible ($iMLS_B$), however, the gene can be expressed constitutively ($cMLS_B$) [3]. In *S. pyogenes* few groups of the *erm* genes have been detected so far – *erm*(A), with its subtype *erm*(TR), *erm*(B), and *erm*(T). The second resistance mechanism relies on the concomitant action of an efflux pump which exports macrolide antibiotics out of the bacterial cell and a protein, which protects the ribosome by driving dissociation of bound macrolide from the ribosome. The associated phenotype called the M phenotype is characterized by low-level resistance to 14- and 15-membered macrolides (e.g., erythromycin and clarithromycin). This type of resistance in GAS is specified by efflux pumps encoded by *mef*A or *mef*E genes, and an *msr*(D)-encoded ribosome protector [4].

Macrolide resistance genes in GAS are frequently located on integrative and conjugative elements (ICEs). ICEs are diverse mobile genetic structures, distributed in virtually all bacterial genera, integrated into chromosomes, that can excise, circularize, and transfer horizontally via conjugation to neighboring bacteria. Upon integration into bacterial chromosomes, ICEs generate directly repeated sequences (DRs) [for review [5]]. ICEs are important players in bacterial evolution and the lateral spread of antibiotic-resistance genes [6,7].

In the last two decades, there has been a gradual increase in the prevalence of macrolide-resistant *S. pyogenes* (MRSP) isolates in Europe, with some countries rising to over 30%; Italy, Spain, and Greece are among the most affected. Recently, the decline of MRSP isolates has been observed in these countries [8–10]. In multiple studies conducted in different parts of the world, clonal proliferation of certain serotypes of GAS, such as M4, M28, M75, and M77 has been observed [8,10–12]. One of the main drivers of macrolide resistance in *S. pyogenes* is the widespread use of macrolide antibiotics for the treatment of respiratory tract infections. The overuse and misuse of antibiotics have led to the emergence and spread of resistant strains, making treatment more difficult and expensive. Continued surveillance and monitoring of MRSP isolates are essential to prevent the spread of resistance and to ensure effective treatment of *S. pyogenes* infections. In Poland, the level of GAS resistance to erythromycin is estimated in the range of 12%, with a tendency to systematic growth [13]. Recent data estimate the prevalence of resistant strains at around 16-18% [14]. Although the incidences of *S. pyogenes* infections are monitored, the data from central Europe on the strain analysis are missing, therefore we hope to fill this gap. Here, we present the genomic analysis of MRSP clones of the M77 serotype collected in Poland between 2003 and 2017.

MATERIAL AND METHODS

***S. pyogenes* strains.** The 136 *S. pyogenes* M77 strains were collected between 2003 and 2017 from patients in two multicenter surveys, BINet and Alexander/RESPI-net [15,16], concerning community-acquired bacterial infections in Poland: 117 were isolated from respiratory tract infections, 3 from skin infections, 1 from urogenital tract infection, and 15 isolates were invasive (with 12 collected from blood; **Supplemental File S1)**. A general description of these strains is presented in Sitkiewicz *et al*. [14] and included as **Supplemental File S1.**

Whole-genome sequencing (WGS). Genomic DNA of all isolates was sequenced using a short read approach with Illumina technology (Illumina Inc., USA)**.** To confirm the structure of mobile genetic elements carrying antibiotic-resistance genes, eight representative strains were selected for the long-read sequencing using Oxford Nanopore technology to obtain complete physical maps. Detailed information regarding the DNA isolation and sequencing strategies are described in **Supplemental File S2**. Sequence reads obtained during this study were deposited in the SRA database under BioProject ID PRJNA1098028. Complete *S. pyogenes* M77 genomes were deposited in the NCBI GenBank database under accession numbers: CP155734-CP155741.

Bioinformatic analysis. Sequence type (ST) and *emm* type were determined using SRST2 v.0.2.0 (https://github.com/katholt/srst2). Sequence annotation was performed using DFAST v.1.2.18 (https://github.com/nigyta/dfast_core). Antimicrobial resistance genes and virulence genes were identified using Abricate v.1.0.0 (https://github.com/tseemann/abricate), based on CARD [17], and VFDB [18] databases, respectively, retaining only hits with >90% identity and >80% target coverage. Core-SNP phylogeny was inferred using snippy v.4.6.0 (https://github.com/tseemann/snippy) including masking of the prophage regions, followed by recombination removal by Gubbins [19] and phylogenetic tree construction using FastTree v.2.1 (http://www.microbesonline.org/fasttree/) with a generalized time-reversible (GTR) model. Pairwise SNPs were calculated using snp-dists v.0.8.2 (https://github.com/tseemann/snp-dists). The data on *S. pyogenes* M77/ST63 strains isolated in other countries available in public databases were included in the phylogenetic analysis (**Supplemental File S3**). MGEs were identified within scaffolded genomes using MobileElementFinder v.1.1.2 [20], ICEfinder [21], VRprofile2 [22], and ICEScreen (https://icescreen.migale.inrae.fr/) tools. Prophage sequence detection was conducted using DEPhT v1.2.2 (https://github.com/chg60/DEPhT) and geNomad v.1.7.5 (https://github.com/apcamargo/genomad); manual inspection applying BLASTn [23] was performed to verify the identification results. The phylogenetic tree and heatmap figures presented in this study were visualized in R v.4.3.3 environment using ggtree v.3.10.1 (https://github.com/YuLab-SMU/ggtree) and ComplexHeatmap v.2.18.0 (https://github.com/jokergoo/ComplexHeatmap) packages, respectively.

RESULTS

Strains characteristics

All 136 GAS with the M77 serotype collected during the 2003–2017 period were tetracycline resistant, majority of them (104, 76.5%) were also erythromycin-resistant. The macrolide resistance phenotype was assayed according to EUCAST (http://www.eucast.org/clinical_breakpoints/); the double disc diffusion test was performed to distinguish between $iMLS_B$ and $cMLS_B$ phenotypes as described by Sitkiewicz *et al.* [14]. Of the erythromycin-resistant strains 93 exhibited $iMLS_B$ phenotype, and 11 strains - $cMLS_B$ [14]. No strains with the M phenotype were detected. The temporal distribution of M77 MRSP collected in Poland over 15 years is presented in **Figure 1**. The total number of M77 GAS, as well as the MRSP isolates, increased from 2011 till 2016, however, we observed a dramatic drop in the number of isolates in 2017, despite a whole year collection of strains. This trend cannot be confirmed as the M77 GAS strains temporal distribution was not analyzed after 2017.

WGS analysis

All 136 strains were analyzed by the short-read WGS approach, and the 8 representative strains bearing different classes of ICEs carrying antibiotic resistance genes, were subjected to long-read

sequencing to confirm the correct assembly of those elements. The resulting genomic characteristics are presented in **Supplemental File S1.** The assembly utilizing long reads is also beneficial in proper genome assembly as the streptococcal chromosomes contain multiple insertion sequences [24] which may affect assembly quality when only short reads are used. Genome alignment of *S. pyogenes* complete chromosomes obtained in this study reveals a high degree of synteny and a high level of nucleotide identity to the complete sequence of *S. pyogenes* NCTC13742 strain (**Supplemental Figure S1**). The summary of analyzed traits, such as year and anatomical site of isolation, presence of antibiotic resistance genes, and ICE content related to the macrolide resistance is presented in **Figure 2**.

All of the M77 strains represented ST63. Previously, the M77/ST63 isolates were also reported in Spain [10], Greece [8], the UK [25], and Iceland [26] as well as in other European countries [27,28], and the US, Australia, New Zealand, and Canada [28].

Antibiotic resistance genes

The tetracycline determinant detected in all strains was the *tet*(O) gene (**Figure 2**), coding for the ribosome protection protein [29]. It is located on an ICE which has 99% nucleotide identity to ICE*Sp2906* described previously for the *S. pyogenes* strains isolated in Italy [6], except for the 6950-bps segment detected in Polish isolates inserted within *orf6* of ICE*Sp2906* (see further description in text). A single strain, NILSPYO771065, was found to harbor in addition to *tet*(O), also *tet*(M), encoding another type of a ribosome protection protein, located within Tn*3872* [30], the Tn*916*-family member.

All MRSP strains carried *erm*(TR) as the macrolide resistance determinant and a single isolate, NILSPYO771065, contained additionally *erm*(B). The *lrm*P gene, coding for a proton motive force-dependent drug transporter [31], was detected in 136 genomes, both in macrolide-sensitive and -resistant strains. Therefore, we assume it did not confer macrolide resistance *in vivo* in the analyzed strains (**Figure 2**). The *mef*A, *mef*E, and *msr*(D) genes were not identified.

Most erythromycin-resistant strains were isolated between 2008 and 2017 (also considered newer strains in the analysis), and are classified predominantly to clade I based on core SNP analysis (see below). Strains isolated before 2008 are either resistant or sensitive to erythromycin belonging mostly to clade II (older strains) (**Figures 2 and 5**).

Virulence genes

Detected GAS virulence factors (VFs) encoded in the core genome, and on prophages are summarized in **Figure 2**. The number of VF genes encoded by a single isolate varies from 14 to 19, with most isolates (approx. 45%) carrying 17 genes. Thirteen VF genes, including *has*ABC coding for the capsule, have been identified in all 136 strains. The least frequent VF gene is *spe*A detected in 2 strains. The VF identification results are presented in detail in **Supplemental File S4.**

Mobile genetic elements

Insertion Sequences, Prophages, and other mges

Sequenced M77 GAS strains are similar to other sequenced strains of other GAS serotypes in terms of MGE content. In the analyzed M77 isolates multiple Insertion Sequences (ISs) were identified, IS*110*, IS*21*, IS*256*, IS*3*, IS*30*, IS*As1*, and IS*L3* (**Supplemental File S5**), all of which are common in streptococcal genomes [32]. The IS element repertoire identified in scaffolded genomes was validated by analyzing the complete chromosomes of eight representative strains. The set of ISs detected was consistent across both scaffolded and complete genomes; however, the copy number of two ISs varied. An additional IS*As1* copy was found in all complete genomes, while an extra IS*3* copy was observed in two genomes (**Supplemental File S5**). These differences were due to the improved resolution provided by the assembly of complete chromosomes utilizing long sequencing reads.

We also identified two transposable elements - Tn*GBS2.3* and Tn*3872* [30,33] (**Supplemental File S6**). Tn*GBS2.3* was identified in group B Streptococci, but it was demonstrated to transfer to GAS via conjugation with relatively high frequency. Tn*3872*, described first in *S. pneumoniae*, belongs to the Tn*916*-family transposons reported initially in *Enterococcus faecalis*, constituting a large family of ICEs, common in *Firmicutes* [for review [34]], that can harbor multiple genes conferring antibiotic resistance.

Interestingly, 96 M77 strains carry RD2 elements identified previously in *S. pyogenes* MGAS6180 and MGAS10270 [35]. This ICE can be transferred via conjugation to multiple GAS serotypes, and to GBS [36]. The RD2 element encodes numerous virulence factors and is considered the major element affecting the colonization potential of GAS strains [37], as it encodes cell surface anchored adhesins and R28 protein.

We detected five prophages integrated into M77 genomes, namely ϕM77.1 to ϕM77.5 (**Figure 3**). Their nucleotide sequences are 96% - 100% identical to known streptococcal phages. Phages ϕM77.1 and ϕM77.3 may be more common for M77 strains, while phage ϕM77.5 is widely spread in multiple GAS serotypes such as M1 or M12 and M77 (detailed analysis in **Supplemental File S7 and S8)**.

Tet(O)- and *erm*(TR)-containing elements

ICESp2905 and its variants

The *tet*(O)-containing element ICE*Sp2906* [6] was detected in all collected M77/ST63 strains. It was either alone, (n=34; in macrolide-sensitive strains n=32), or as a hybrid element with IME*Sp2907* (n=27), an Integrative Mobilizable Element, constituting ICE*Sp2905* [6](**Figure 4A**). ICE*Sp2906* was also detected as a hybrid with an ICE*Sp1108*-like element (n=75) forming a novel element, ICE*Sp1109* (see below). Both ICE*Sp2905* and ICE*Sp1108* [7], are integrated into the

M77/ST63 strains genomes between the *rum* and *pnp* genes, coding for the 23S rRNA m(5)U(1939)methyltransferase and a phosphorylase superfamily protein [6], respectively. Such integration is catalyzed by a site-specific serine integrase, encoded by a gene located at the 3' ICE proximity [38].

In MRSP isolates, IME*Sp2907* was the source of the *erm*(TR) gene similarly to what was detected in *S. pyogenes* in Italy [6,7]. In three strains, NILSPYO770002, NILSPYO770111, and NILSPYO771066, besides ICE*Sp2906* another ICE bearing *erm*(TR) was detected, with sequence blocks identical in 94-100% to ICE*Sp1108,* and the two mentioned ICEs were located in the genome approx. 400 kbps apart from each other (**Figure 4B**). It should be stressed that compared to the original ICE*Sp2906* sequence [6], the 5' terminus of ICE*Sp2906* of the three mentioned strains contains an insertion of 6950-bps sequence comprising 6 additional *orf*s. The extended ICE*Sp2906* with such an insertion was identified in all Polish *S. pyogenes* M77/ST63 strains. Moreover, a structure identical in 99% to the modified ICE*Sp2906* element was also detected in the *S. pyogenes* NCTC13742 (Acc. No. LS483386.1), and *S. pyogenes* TSPY453 (Acc. No. CP033337.1) genomes; these are also M77/ST63 strains and were isolated in 2015 in the United Kingdom and 2014 in the USA, respectively. The origin of the entire 6950-bps fragment is unknown, although fragments identical in 86-94% to some parts of this sequence (coverage 78-92%) could be detected in various streptococci: *Streptococcus. equi subsp. zooepidemicus* (strains SEZ33, SEZ25, NCTC12090 and others), *Streptococcus. dysgalactiae subsp. equisimilis* 89, *Streptococcus suis,* but also in the genomes of other bacteria related to the human microbiome such as *Filifactor alocis* ATCC 35896, or *Aerococcus* species.

The ICE*Sp2905* element was identified in 27 isolates collected predominantly in the first years of the analyzed period. Interestingly, the canonical version of this element, identical (99-100%) to the sequence deposited in the EMBL database under Acc. No. FR691055 [7], was detected in 12 strains while other strains carry the ICE*Sp2905* variants with an insertion within *orf6* of this element (**Figure 4A**). The localization and the composition of individual elements in the representative strains, NILSPYO771004 with ICE*Sp2905*, and NILSPYO770020 with the ICE*Sp2905* variant were confirmed by *de novo* assembling of their complete chromosomes.

It is worth noticing that in the case of three strains, NILSPYO770002 (complete genome assembly), NILSPYO770111 (scaffolded genome), and NILSPYO771066 (scaffolded genome), with distantly located ICE*Sp2906* and the ICE*Sp1108*-like elements, ICE*Sp2906* is deprived of its terminal sequences, at the 5' end, and only the terminal 43-bps *rum* fragment is present, the 3' end is truncated by the 1055 bps-DNA fragment including entire *pnp* together with terminal 36 bps of the preceding gene (**Figure 4B**).

ICESp1109, a novel hybrid element

In the majority of strains collected between 2008 and 2017 (n=75), the ICE*Sp1108*-like element (94-99% nucleotide identity) was inserted in the very 5' flank of ICE*Sp2906* giving rise to a novel

hybrid element which we named ICE*Sp1109* (**Figure 4C, Supplemental File S9**). Similarly to ICE*Sp2905* and ICE*Sp1108*, ICE*Sp1109* integrated between the *rum* and *pnp* genes. The genomes of the first collected strains comprising ICE*Sp1109*, NILSPYO771038, and NILSPYO771039, and the last one NILSPYO770049, were assembled into complete chromosomes. So far, the ICE*Sp1109* hybrid element has not been described nor deposited in available public databases. Direct repeats (DRs) also known as attachment sites, left and right, (*att*L and *att*R) generated upon ICE integration were detected in NILSPYO771038 for both ICE*Sp1109* components, ICE*Sp1108*-like and ICE*Sp2906* variant [7]. The putative sequences, *att*L and *att*R of the ICE*Sp1108*-like element were identified within the 3' end of the *rum* gene and in the serine recombinase gene, respectively (**Supplemental Figure S2**). The *att*L sequence of ICE*Sp2906* was detected to overlap *att*R of the ICE*Sp1108*-like element, and *att*R of ICE*Sp2906* within ICE*Sp2906* serine recombinase gene (**Supplemental Figure S2**). The experimental data are required to determine whether ICE*Sp1109* is active as an entire element.

Tn3872, a tet(M)- and erm(B)-containing element

In the genome of the single strain, NILSPYO771065, besides the *erm*(TR) and *tet*(O) genes detected within ICE*Sp1109*, we also identified *erm*(B) and *tet*(M), located on Tn*3872* (**Figure 4D**), which is part of a larger element of the Tn*5252* superfamily, over 62 kb in size (**Supplemental Figure S3**, detailed analysis in **Supplemental File S10**).

Phylogenetic analysis of M77/ST63 strains

Most M77 strains isolated in Poland and worldwide (over 60% according to PubMLST database (https://pubmlst.org/organisms?title=Streptococcus+pyogenes) are classified as ST63. NILSPYO771001, the first complete genome of the erythromycin-sensitive Polish M77 *S. pyogenes* strain was used as a reference genome for the Single Nucleotide Polymorphism (SNP) tree construction. The strain was isolated in 2003, i. e. earlier than the NCTC13742 type strain, which was collected in 2015. The genomes of the Polish M77/ST63 isolates (n=136) were compared to M77/ST63 isolates (n=253) from the United Kingdom, Iceland, Spain, Germany, France, Sweden, Canada, Australia, New Zealand, and the US, whose genomes were available in public databases (accessed on 1st March 2024) (**Figure 5**) [10,25,26]. A total of 1344 core genome SNPs were identified in the tested dataset that included strains isolated worldwide. The number of polymorphic sites detected among M77 strains is higher than observed among highly clonal serotypes such as M1 or M3, where differences do not exceed 100 SNPs [39]. The Polish M77 isolates had a maximal pairwise SNP difference of 76 (average difference of 22 core SNPs), and when all M77 isolates were included, the maximal pairwise SNP difference increased to 87 (average difference of 38 core SNPs) (**Supplemental File S11**).

The majority of Polish strains form two separated clusters with clonal distribution (clades) (**Figure 5**). One of them, Clade I, groups all the isolates containing *erm(TR)* within the ICESp*1109* hybrid element (n=75), NILSPYO771066, NILSPYO770111, and NILSPYO770002 – the three clones

with the distantly inserted ICE*Sp2906* and *ICESp1108*-like elements in their chromosomes. The first strain (NILSPYO771038) with identified ICE*Sp1109*, was isolated in 2008 and in the 2013 – 2017 period this clone dominated the population of the Polish MRSP strains. In total, 487 core genome SNPs were detected among Clade I strains and a maximal pairwise SNP difference of 25 was identified (**Supplemental File S11**). The Clade II contains NILSPYO771001 reference strain and groups the majority of erythromycin-sensitive older clones (with ICE*Sp2906*), and 7 of 27 carrying *erm*(TR) within the ICE*Sp2905* variant element both in older (from 2003 to 2008) and newer (from 2008 to 2017) strains. In total, 235 core genome SNPs were identified in Clade II, with a maximal pairwise SNP difference of 20.

The average pairwise difference of 10 core SNPs in Clade I and 8 core SNPs in Clade II (**Supplemental File S11**), respectively, indicate their clonal distribution.

Other Polish isolates with ICE*Sp2905* or its variant and a few carrying ICE*Sp2906* cluster predominantly with European isolates (from the UK, Spain, and Iceland) and also with several strains originated from the USA, isolated in 2016 and 2017. The majority of the strains isolated in Europe (the UK, Iceland, Spain, Germany) are more diverse than those isolated in the USA, which are clonally distributed.

There were no differences between clusters identified by core SNP analysis with strains NILSPYO771001 or NCTC13742 as a reference (**Supplemental Figure S4**). With *S. pyogenes* NCTC13742 as a reference, 1339 core genome SNPs were detected among all tested strains. All Polish isolates (n=136) and a majority of the isolates from other countries (n=237) carry the *tet*(O) gene within the ICE*Sp2906* element. No correlation was found between the SNP composition in analyzed strains and their ICE repertoire (**Supplemental File S11**).

DISCUSSION

The *S. pyogenes* M77 isolates collected in Poland in 2003-2017 represent a single ST63, which has also been reported in other European countries [8,10,25–28], and North America, Australia, and New Zealand [28]. Our phylogenetic analysis of M77/ST63 strains shows relationships between strains isolated in different parts of the globe suggesting clonal dissemination of analyzed strains. Unfortunately, our observations are limited due to the lack of whole genome sequencing data from other European countries, especially from central Europe. In public databases, there was no data from Poland's neighboring countries, such as the Czech Republic, Slovakia, or the eastern part of Europe, and the limited dataset was available from Germany (2 isolates).

The erythromycin-resistant Polish M77/ST63 isolates carry the *erm*(TR) gene within the *tet*(O)-containing ICE elements. In the early isolates *erm*(TR) is found in the already known ICE*Sp2905* [6] or its variant. Starting from 2008 it is located in ICE*Sp1109*, the novel hybrid element described in this work, composed of the ICE*Sp2906* variant and ICE*Sp1108*-like elements. Although most

M77/ST63 strains were analyzed as draft genomic sequences, the genomic organization of MGEs was confirmed by assembling the complete genomes of selected isolates using long-read sequencing. No isolates with *erm*(TR) without *tet*(O) were detected, suggesting that ICE*Sp2906* acquisition was a prerequisite for *erm*(TR) gaining. The strains carrying the ICE*Sp1109* element disseminated clonally in Poland and dominated the population of *S. pyogenes* M77/ST63. Due to the lack of sequence data from neighboring countries, we cannot determine whether such a clone is disseminated elsewhere.

The surveillance and monitoring of macrolide-resistant *S. pyogenes* have to be ongoing due to the public health challenges underlined recently also by the World Health Organization in 2024 which added these bacteria to the medium-priority group of pathogens [40].

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Compliance with Ethics Guidelines: The study was conducted as continuous surveillance following the World Health Medical Association 1966 Declaration of Helsinki and the EU rules of Good Clinical Practice.

Potential Conflict of Interest: All authors declare that they have no conflicts of interest.

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Figure 1 Temporal distribution of M77/ST63 MRSP isolates in Poland between 2003 and 2017. MRSP – macrolide-resistant *S. pyogenes.*

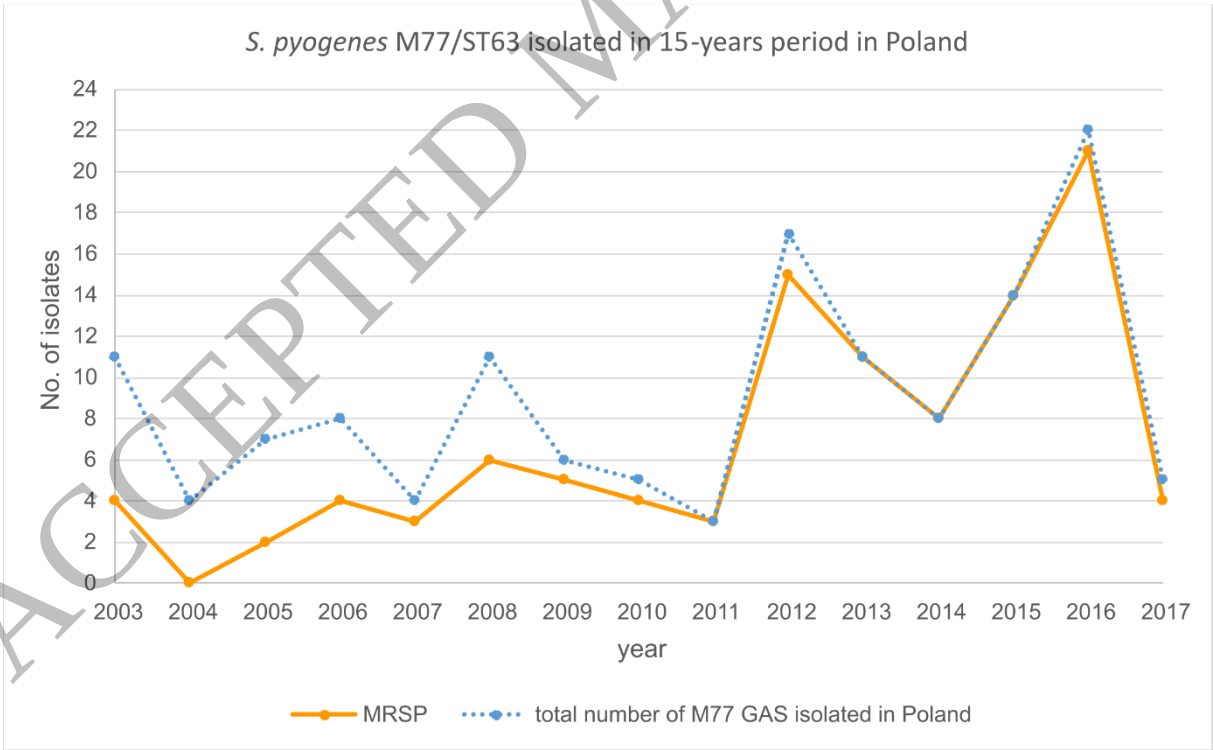

Figure 2 Presence/absence matrix depicting the antibiotic resistance and virulence genes repertoire detected in *S. pyogenes* M77 isolates. The coloured squares represent: the first column on the left side *the year of sample isolation,* the second - the strain isolation site, and the third - describes the type of *erm*(TR) containing ICE. The red squares represent the presence of resistance determinants of macrolides [*erm*(TR), *erm*(B), *lmr*P], and tetracyclines [*tet*(M) and *tet*(O)]. The blue squares represent a presence/absence matrix of virulence factors.

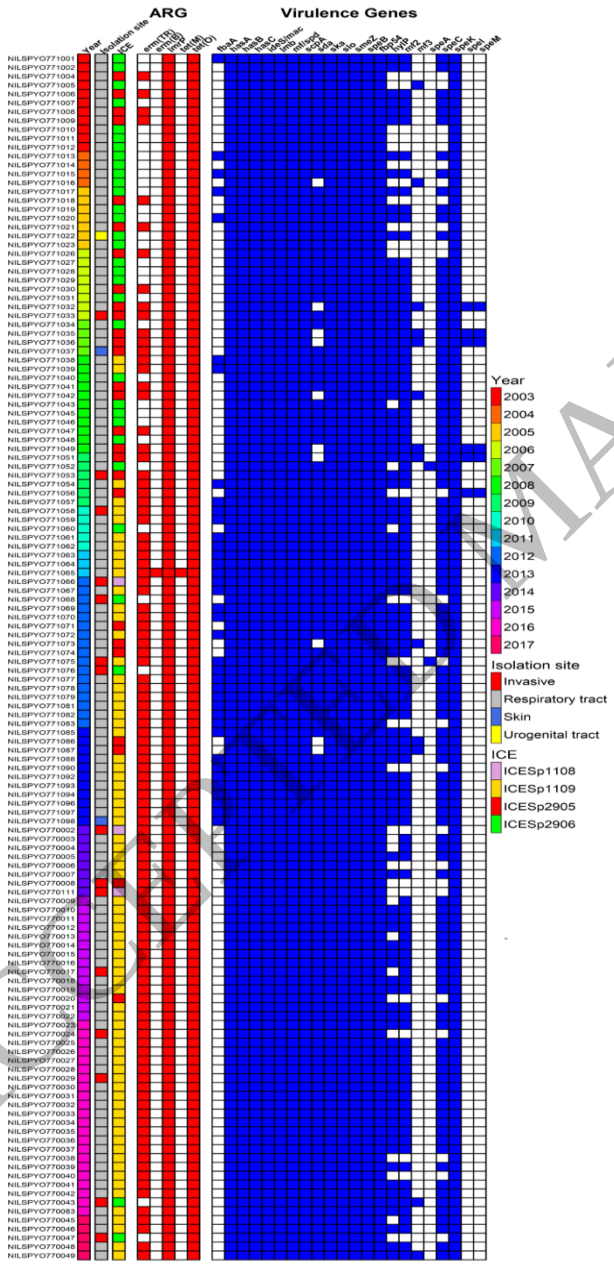

Figure 3 Schematic overview of the Polish *S. pyogenes* M77/ST63 core chromosome, prophage element insertion sites, and Integrative and Conjugative Elements (ICEs). The circle represents the GAS chromosome with the marked nucleotide positions and the origin of replication *(ori).* The prophage elements are indicated with green triangles and ICEs as squares that are color-coded according to the ICE type; stacked squares - ICEs inserted at the same site. Blue letters denote the integration sites, described in the right panel. The presence of the given element in the sequenced GAS genomes is indicated.

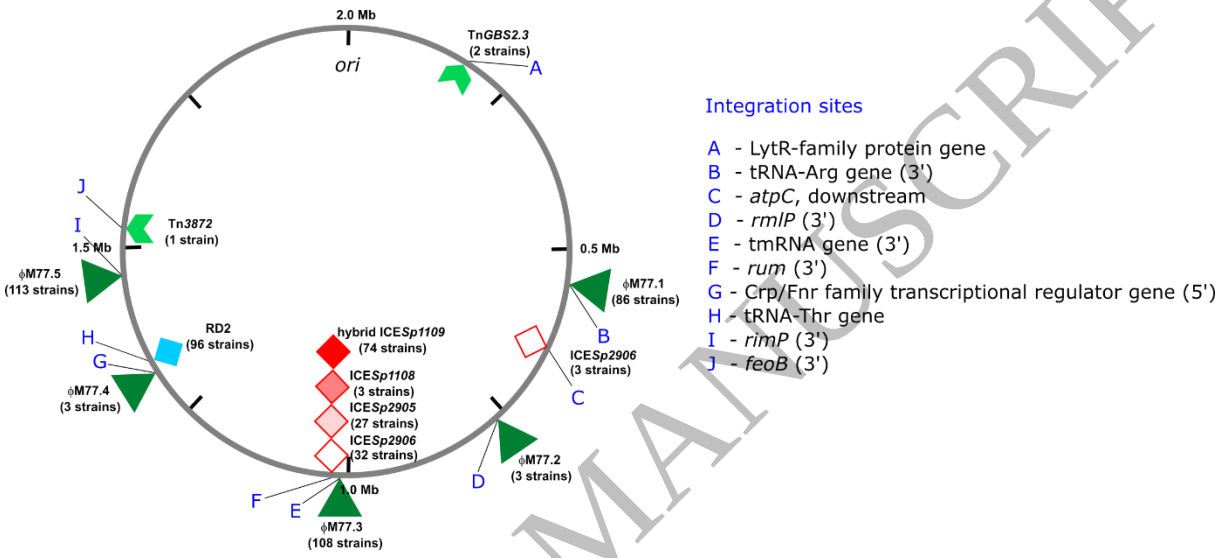

Figure 4 Schematic representation of the *erm*(TR)-containing Integrative and Conjugative Elements identified in the genomes of *S. pyogenes* M77/ST63 strains: **A/** ICE*Sp2905* in the NILSPYO771004 genome and the ICE*Sp2905* variant in the NILSPYO770020 genome; **B/** The *tet*(O)- and *erm*(TR)-containing ICEs in the NILSPYO770002, NILSPYO770111, and NILSPYO771066 genomes. The genomic location of the specific fragment is marked; **C/** ICE*Sp1109*, the novel *erm*(TR)-*tet*(O)-containing hybrid element present in the NILSPYO771038 strain; **D/** Tn*3872* of the NILSPYO1065 strain, comprising the *tet*(M) and *erm*(B) genes. Tn*3872* (GenBank Acc. No. OP715845.1) and Tn*916* (GenBank Acc. No. U09422) are shown. The functional modules of Tn*916* are marked: conjugation, regulation, recombination, and the accessory gene *tet*(M). Antibiotic resistance genes are shown as red arrows. The *rum* and *pnp* genes are marked as light green arrows.

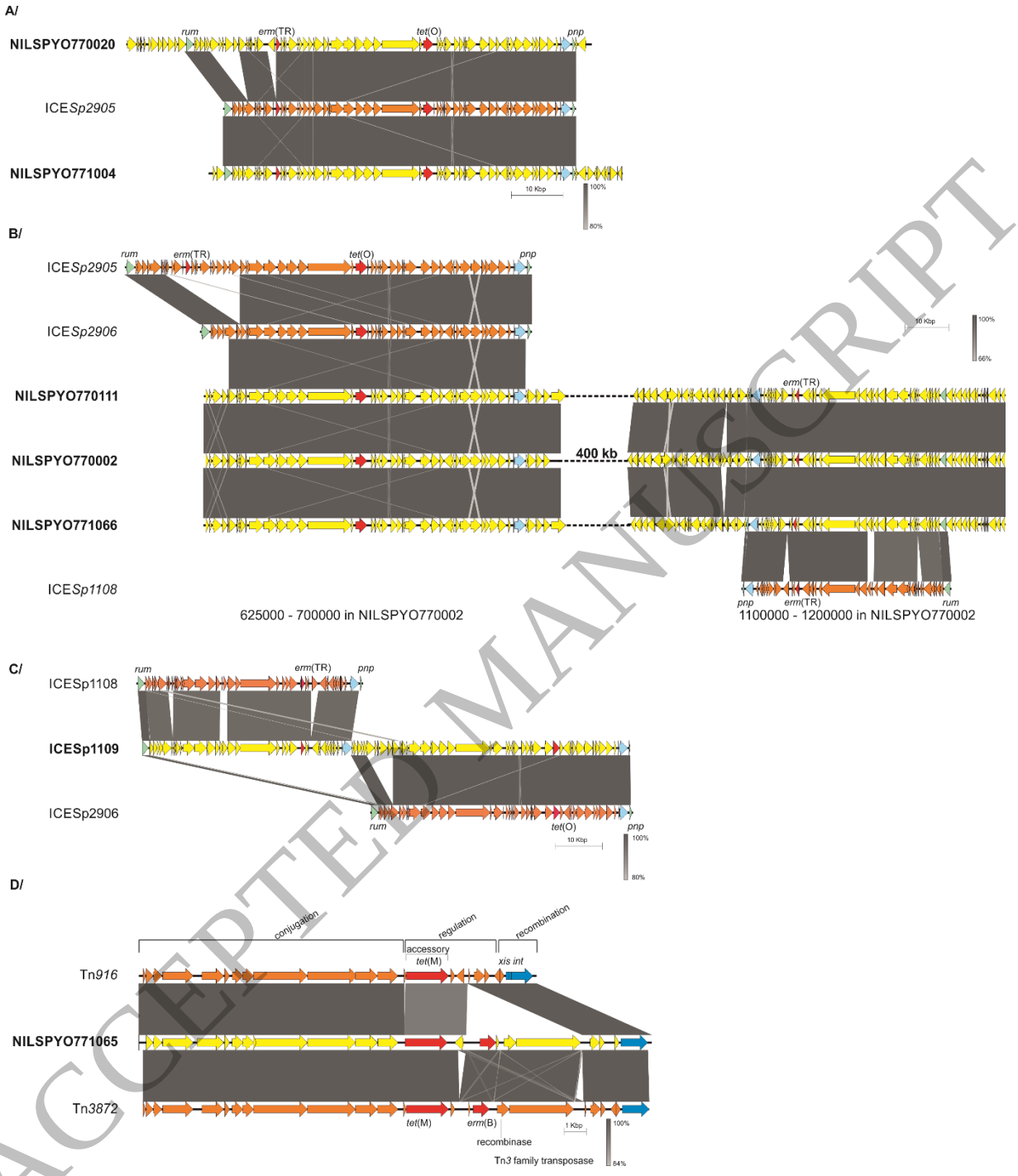

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Figure 5 The maximum-likelihood core-SNP phylogenetic tree of 389 *S. pyogenes* M77/ST63 isolates including 136 Polish isolates, and strains from other countries (BioProjects accession numbers indicated in **Supplemental File S3**). The innermost ring denotes *erm*(TR)-presence or -absence, the second ring – the relevant ICE element presence in the strain, and in the third ring, the year of sample isolation is presented. The clades representing two major groups of Polish isolates analyzed in this study are marked in the outermost ring. The NILSPYO771001 genome was used as a reference sequence.