Clinical Microbiology and Infection 28 (2022) 1499.e7–1499.e14

Contents lists available at ScienceDirect

Clinical Microbiology and Infection

journal homepage: www.clinicalmicrobiologyandinfection.com

Original article

The emergence of a multidrug resistant *Salmonella* Muenchen in Israel is associated with horizontal acquisition of the epidemic pESI plasmid

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ARTICLE INFO

Article history:
Received 13 December 2021
Received in revised form
19 May 2022
Accepted 23 May 2022
Available online 30 May 2022

Editor: S. Malhotra-Kumar

Keywords:
Antibiotic resistance
Conjugation
Emerging pathogens
Epidemiology
MDR
Outbreak
pESI
Salmonella Muenchen
Salmonellosis

ABSTRACT

Objectives: Horizontal acquisition of mobile genetic elements is a powerful evolutionary driving force that can profoundly affect pathogens epidemiology and their interactions with the environment and host. In the last decade, the role of the epidemic megaplasmid, pESI was demonstrated in the global emergence of multi-drug resistant (MDR) *Salmonella enterica* serovar Infantis strains, but it was unknown if this was a one-time phenomenon, or that pESI can drive the emergence of other pathogens.
Methods: Epidemiological, molecular, whole genome sequencing, de-novo assembly, bioinformatics and genetic approaches were used to analyze the emergence of a pESI-positive *Salmonella enterica* serovar Muenchen strain in Israel.
Results: Since 2018, we report the emergence and high prevalence of *S.* Muenchen in Israel, which consisted at 2020, 40% (1055/2671) of all clinical *Salmonella* isolates. We show that the emergence of *S.* Muenchen is dominated by a clonal MDR strain, report its complete assembled genome sequence, and demonstrate that in contrast to preemergent strains, it harbors the epidemic megaplasmid, pESI, which can be self-mobilized into *E. coli* and other *Salmonella* serovars. Additionally, we identified bioinformatically highly similar genomes of clinical isolates that were recently collected in South Africa, UK and USA.
Conclusions: This is a second documented case of a pathogen emergence associated with pESI acquisition. Considering the genetic cargo of pESI that enhances resistance, stress tolerance and virulence, and its ability to conjugate into prevalent *Salmonella* serovars, we provide further support that pESI facilities the emergence and spreading of new *Salmonella* strains. **Emiliano Cohen, Clin Microbiol Infect 2022;28:1499.e7–1499.e14**

Introduction

The ubiquitous bacterial species *Salmonella enterica* is a facultative intracellular animal and human pathogen, and a leading source of foodborne infections, both at industrialized and developing countries [1]. This highly diverse species consists of more than 2600 antigenically-distinct serovars that can cause different clinical manifestations in a wide range of hosts [2]. Human infection can lead to different clinical outcomes, ranging from asymptomatic infection, acute self-limited enterocolitis, invasive systemic salmonellosis and bloodstream infection, to a life-threatening disease known as enteric or typhoid fever [3]. *S. enterica* infections are still a significant cause of morbidity and mortality with an annual incidence of over 27 million cases of enteric fever [4] and 78.7 million cases of gastroenteritis [5] worldwide.

Global epidemiology of *Salmonella* is largely shaped by the emergence of dominant antibiotic resistance clones and their international dissemination. For example we can mention the worldwide spread of the multidrug resistant (MDR) *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) DT104 during the

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https://doi.org/10.1016/j.cmi.2022.05.029

1990s [6], or the dissemination of the MDR *S.* Kentucky ST198 during the early 2000s [7].

More recently, the emergence of MDR *S.* Infantis was reported in many regions of the world including Israel [8], Europe [9–13], UK [14], Russia [15], South America [16–18], and USA [19]. Different lineages of emerging *S.* Infantis have acquired derivatives of the virulence-resistance megaplasmid termed 'plasmid of emerging *S.* Infantis' (pESI) [8]. This conjugative plasmid was first identified and characterized in an emerging *S.* Infantis clone in Israel and was shown to encode genes providing resistance to tetracycline, sulphonamides, and trimethoprim, and additional virulence factors that could enhance pathogenicity phenotypes and stress tolerance [8,20,21]. Later, conserved pESI-like plasmids were also found in emerging *S.* Infantis lineages worldwide [10,11,13,15–18]. These pESI-related plasmids share the same backbone as pESI, but may carry varying antibiotic resistant cassettes including extended-spectrum β-lactamase (ESBL) genes [9,11,14,19,22]. The reason for the successful spread of pESI-containing clones is not fully understood, however the current view is that pESI contributes to the dissemination of these lineages in the poultry industry and increases the fitness of its bacterial carriers, both in the environment and in the host [8,19,22–24].

Previously we hypothesized that due to the conjugative nature of pESI and its integrated addiction (toxin-antitoxin) systems, this mobile genetic element will be disseminated from *S.* Infantis to other *Salmonella* serovars or additional bacterial species [8,25]. Here, we report the emergence of an MDR *S.* Muenchen strain in Israel that has acquired pESI and its identification using bioinformatics approaches in clinical samples that were recently collected in USA, UK and South Africa.

Methods

***S.* Muenchen epidemiology.** The fraction of *S.* Muenchen from all *salmonella* isolates in Israel is listed in Supplementary Material Table S1 and was inferred from the annual reports routinely published by the Public Health Laboratories, Ministry of Health (https://www.health.gov.il/UnitsOffice/HD/PH/LabDept/PublicHealthLabs/jerusalem_Labs/Pages/annual_reports.aspx) that receive, serotype and document *Salmonella* samples isolated in Israel from clinical, poultry and food sources and uses the Kauffmann–White-Le-Minor scheme for serovar typing.

Bacterial strains and growth conditions. *S.* Muenchen strains (N = 20) used in this study are listed in Supplementary Material Table S2. Bacterial cultures were routinely grown in Lennox Luria-Bertani (LB; BD Difco) medium at 37°C. Antimicrobial susceptibility testing was carried out by the disk diffusion method following the Clinical and Laboratory Standards Institute (CLSI) M100 Performance Standard for Antimicrobial Susceptibility Testing 2021. Antibiotic amounts and cut off that were uses are as follow: Ampicillin 10μg (S ≥ 17 mm); Tetracycline 30 μg (S ≥ 15 mm); Trimethoprim/Sulfamethoxazole 1.25/23.75 μg (S ≥ 16 mm) (R ≤ 12mm); Ofloxacin 5 μg (S ≥ 16 mm); Ciprofloxacin 5 μg (S ≥ 26 mm); Gentamycin 10 μg (S ≥ 15 mm); and Cefpodoxime 10 μg (S ≥ 21 mm). Resistance to Trimethoprim (50 μg/ml), sulfamethoxazole (50 μg/ml), and Streptomycin (50 μg/ml) were tested by direct plating on LB-agar plates and evaluation of bacterial growth.

Whole genome sequencing (WGS), assembly and bioinformatics. WGS and genome assembly of an emergent strain *S.* Muenchen that was isolated from the blood of a patient with bacteremia at 2018 (isolate 180135033, Table S2) was performed as recently explained in [26] and is further detailed in the Supplementary Material.

Sequence identity between pESI-related plasmids was determined by the EMBOSS Matcher tool (https://www.ebi.ac.uk/Tools/psa/emboss_matcher/). To identify genetically-related *S.* Muenchen isolates that may carry pESI, we used the NCBI's Isolate Browser (https://www.ncbi.nlm.nih.gov/pathogens/isolates/) to search for *S.* Muenchen genomes that harbor similar resistance genes as isolate 180135033.

S1 nuclease digestion and PFGE analysis. Plasmid profiling was determined as was recently published [27] and is further detailed in the Supplementary Material.

pESI profiling using PCR. The primers hyp_pESI_Fwd (5′ GCGGTGAAGATGGTTATCAG 3′) and hyp_pESI_Rev (5′ GTGGTAGTT TGTCCTTTGGC 3′) were used to amplify a 559 bp PESI-encoded gene. The PCR was carried out using the Red Load Taq Master mix (LAROVA GmbH). The reaction conditions included a denaturation step at 94°C for 2 minutes; 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds; and a final elongation step for 2 minutes at 72°C.

Calculating pESI conjugation frequency. pESI conjugation was determined using *S.* Infantis 119944 as a donor and 23 *Salmonella* strains from 11 different serovars and *E. coli* ORN172 that carries kanamycin resistance as recipients. All 23 *Salmonella* recipient isolates were first transformed with pWSK129 that confers kanamycin resistance to allow selection of transconjugants. Conjugation was performed as recently detailed [27]. Serial dilutions were plated on LB agar plates supplemented with tetracycline (to select for pESI) and kanamycin (to select for the recipient strains). Conjugation frequencies were calculated as the ratio between the number of transconjugants obtained (CFUs) and the number of donor CFUs. Serial dilution of the donor and the recipient cultures only were also plated on selective plates to exclude events of spontaneous resistance.

Results

S. Muenchen is an emerging MDR pathogen in Israel

Based on the annual reports, routinely published by the Public Health Laboratories in Israel, between the years 2000 and 2017, *S.* Muenchen was identified in only a small fraction of human, poultry or food *Salmonella*-confirmed samples and was responsible for a low number of human salmonellosis cases. In fact, during this period (with the exception of years 2013 and 2014), *S.* Muenchen consisted less than 5% from all Salmonellae identified in Israel (Fig. 1 and Supplementary Material Table S1), positioning *S.* Muenchen rather low in the prevalence order of *Salmonella* serovars. Nevertheless, *S.* Muenchen occurrence has sharply changed in a statistically significant manner at 2018, when the annual proportion of *S.* Muenchen from all *Salmonella* serovars has reached 15% (563/3801). Since 2019, *S.* Muenchen is the most dominant serovar isolated in Israel from clinical, poultry and food sources, responsible for more than 30% (937/3112) and 39.5% (1055/2671) of all *Salmonella* isolates from clinical source at 2019 and 2020, respectively (Fig. 1 and Supplementary Material Table S1). These data indicate that since 2018, *S.* Muenchen is an emerging pathogen in Israel that dominates the national *Salmonella* epidemiology.

Interestingly, testing the antibiotic resistance profile of emergent and preemergent *S.* Muenchen isolates has shown that emergent *S.* Muenchen strains, that were isolated at 2018 or later are multidrug resistant and present a conserved resistance pattern to tetracycline, trimethoprim-sulfamethoxazole, and streptomycin. In contrast, none of the preemergent strains (isolated prior to 2018) tested demonstrated this resistance pattern (Supplementary Material Table S2). Moreover, molecular typing using pulsed-field gel electrophoresis (PFGE) indicated that the emergent *S.* Muenchen isolates, collected between 2018 and 2021 are all similar on the genetic level, but noticeably distinct from preemergent isolates (Fig. 2).

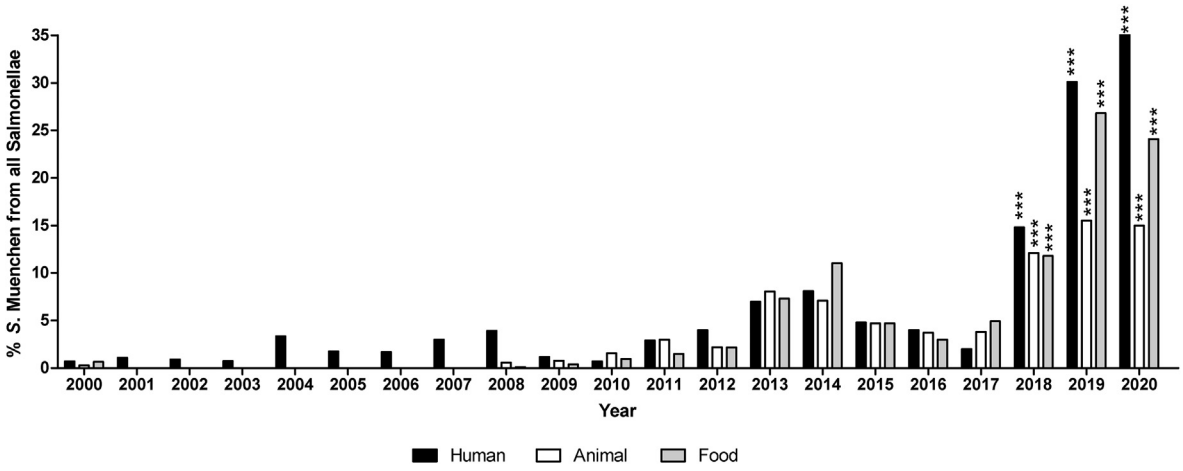

Fig. 1. *S.* Muenchen prevalence, Israel, 2000-2020. The annual relative prevalence of *S.* Muenchen isolates from all *Salmonella* serovars identified at the national *Salmonella* reference center in Israel from human, animal and food sources is shown. Data were inferred from the published annual reports of the national *Salmonella* reference center (https://www.health.gov.il/UnitsOffice/HD/PH/LabDept/PublicHealthLabs/jerusalem_Labs/Pages/annual_reports.aspx). The statistical significance of the difference between the prevalence of *S.* Muenchen (from all *Salmonell*a isolates) at 2018, 2019, and 2020 and its prevalence at 2000-2017 was calculated by the Z Score for two population proportions. ***. $P < 0.001$.

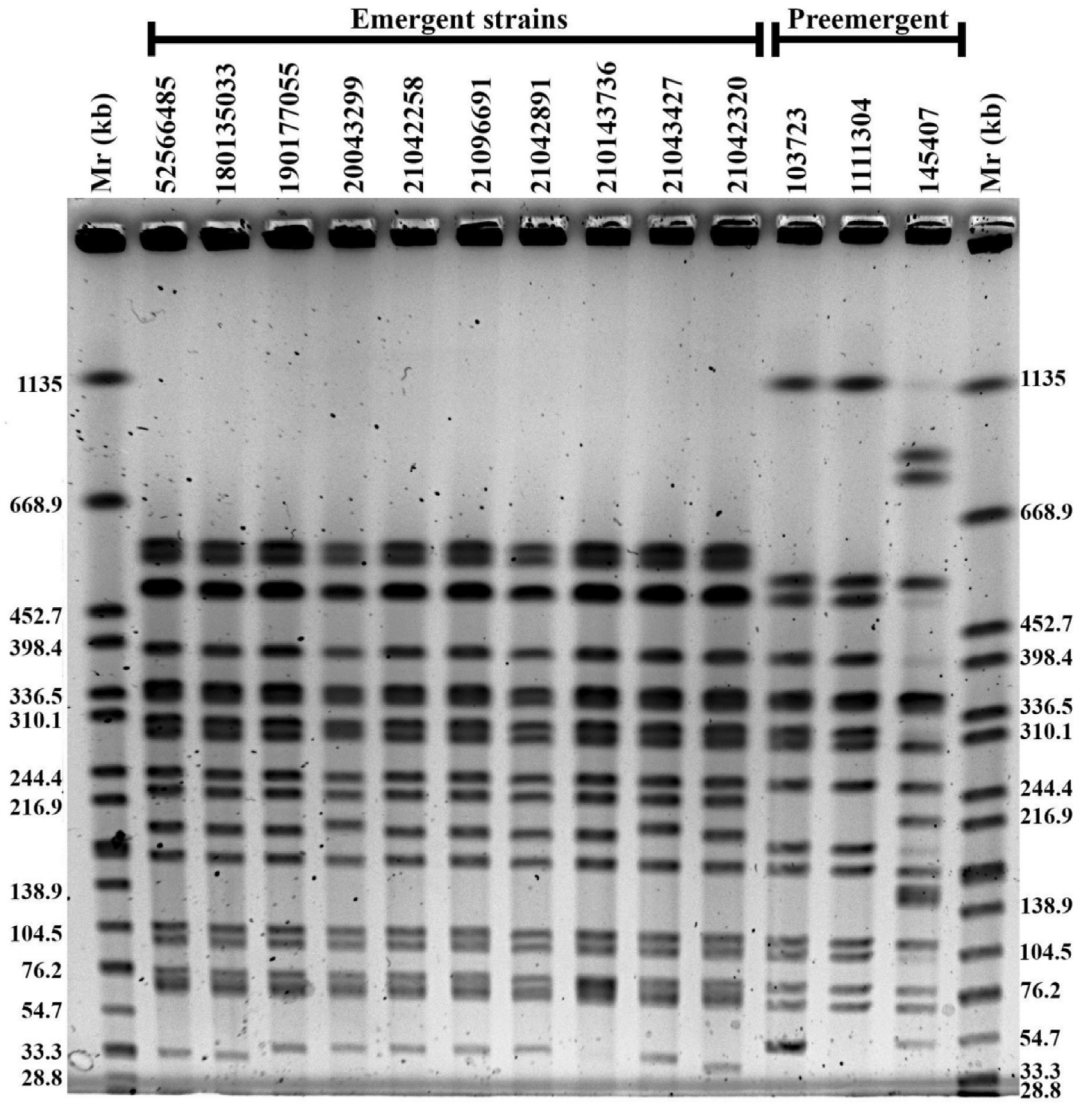

Fig. 2. Emerging *S.* Muenchen isolates are genetically similar and different from preemergent strains. Ten emergent and three randomly selected preemergent *S.* Muenchen isolates were subjected to PFGE analysis using macrorestriction fingerprinting with XbaI.

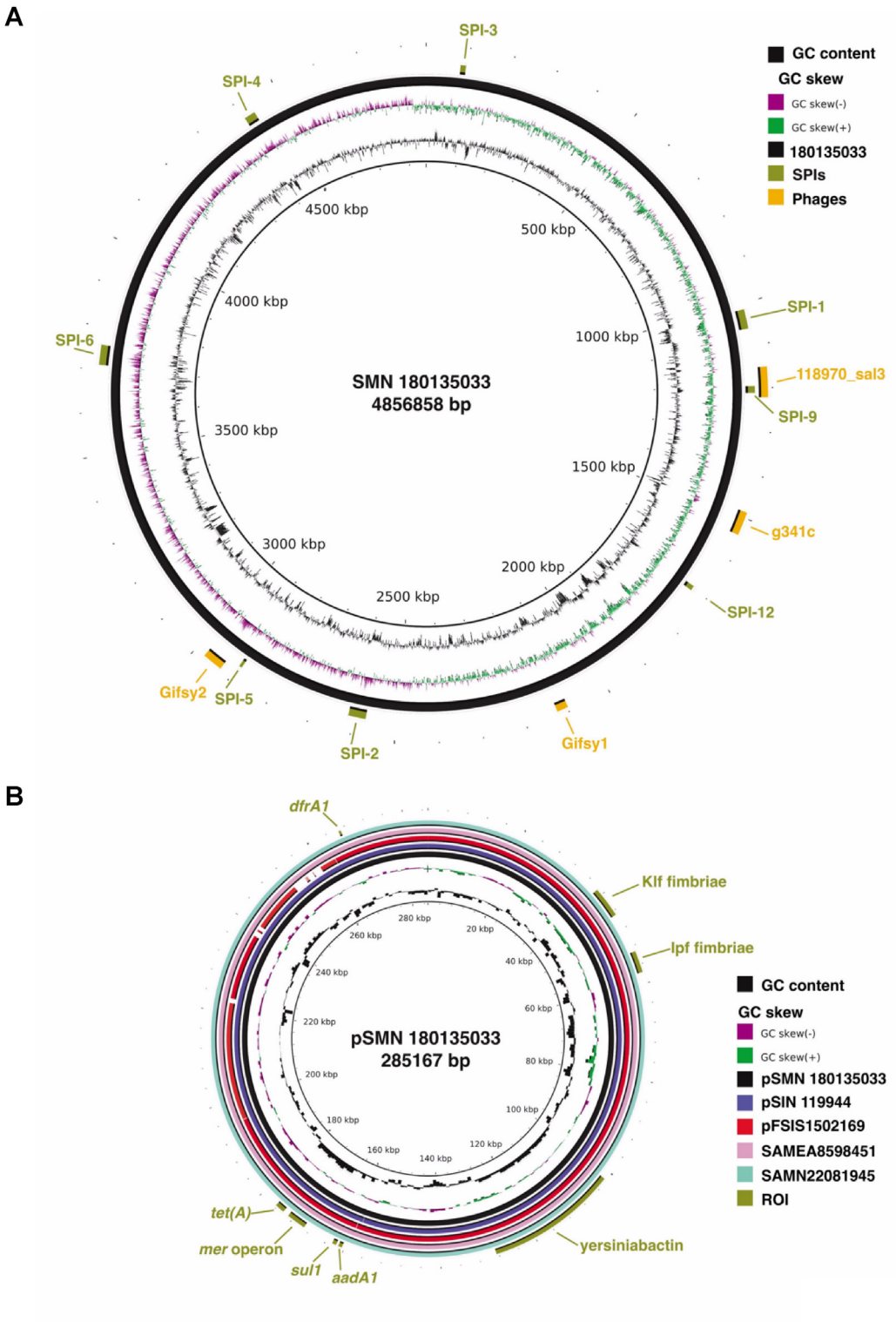

Fig. 3. The genome of an emergent *S.* Muenchen strain and its epidemic plasmid. (**A**) A graphical representation of the chromosome of *S.* Muenchen isolate 180135033 was created by BLAST Ring Image Generator (BRIG). The presence and location of pathogenicity islands are shown in olive green and the location of integrated prophages is shown in yellow. The chromosome sequence of this isolate was deposited at NCBI under accession number CP088901. (**B**) Graphical representation of the *S.* Muenchen 180135033 plasmid (accession number CP088902) is illustrated by the black ring. Sequence comparison with pESI (of *S.* Infantis 119944; red ring; accession number NZ_CP047882) and with pESI-like plasmid of *S.* Infantis strain FSIS1502169 (purple ring; accession number NZ_CP016407) was performed with BRIG. The homology of the *S.* Muenchen pESI with the correspondent assembled sequences of an American (SAMN22081945) and a South African (Accession: SAMEA8598451) *S.* Muenchen isolates are shown by the turquoise and pink rings, respectively. The presence and location of regions of interest (ROI), including antibiotic resistance and main virulence genes are indicated in olive green.

The emerging S. Muenchen harbors the epidemic plasmid pESI

Based on the combined resistance profile of the emergent strain to tetracycline, sulfonamide and trimethoprim, we hypothesized that emergent *S.* Muenchen strains may have acquired pESI. To test this possibility, we performed plasmid profiling for 10 emergent (isolated at 2018 and later) and 10 preemergent (isolated before 2016) *S.* Muenchen isolates by S1 nuclease digest followed by PFGE. This analysis indicated that all of the emergent isolates harbor a 285 Kb plasmid, with the same size as pESI that was absent from all of the preemergent strains (Supplementary Material Fig. S1A). Using specific primers from a pESI-encoded gene, we established that while all of the emergent *S.* Muenchen isolates harbor a putative pESI plasmid, none of the preemergent isolates carries this extra-chromosomal element (Fig. S1B).

Whole genome sequencing and assembly of the emergent S. Muenchen strain

To further characterized the emergent *S.* Muenchen strain, we applied whole genome sequencing and hybrid assembly, while combining short reads from Illumina sequencing together with long reads generated by a MinION platform. Using this approach, we were able to assemble a complete, gap-free genome sequence of *S.* Muenchen isolate 180135033 that was covered 2700 × . The complete genome of the emergent *S.* Muenchen has a GC content of 52 % and comprises of one circular 4,856,858 bp chromosome (Fig. 3A; accession number CP088901) and a single 285,167 bp plasmid (Fig. 3B; accession number CP088902).

Using the NCBI prokaryotic genome annotation pipeline (PGAP), we found that the chromosome of *S.* Muenchen 180135033 encodes 4749 genes, 4630 CDS, 84 tRNA genes, and harbors108 putative pseudogenes. The genome of *S.* Muenchen encodes eight pathogenicity islands (SPIs 1-6, 9 and 12) and carries four intact lysogenic prophages including Gifsy1, Gifsy2, g341c (NC_013059.1), and 118970_sal3 (NC_031940) (Fig. 3A).

Pairwise comparison between the *S.* Infantis 119944 pESI [21] and the plasmid of *S.* Muenchen 180135033 indicated that both plasmids share 285,059 bp (99.96 %) identical sequence. The *S.* Muenchen plasmid is also highly similar to a 323,122 bp ESBL-encoded pESI-like plasmid (pFSIS1502169) that was found in a CTX-M-65-producing *S.* Infantis isolate in USA (NZ_CP016407.1), sharing 250, 551 bp with 96.10% identity (Fig. 3B). Like in *S.* Infantis, the *S.* Muenchen's pESI harbors a wide array of resistance genes

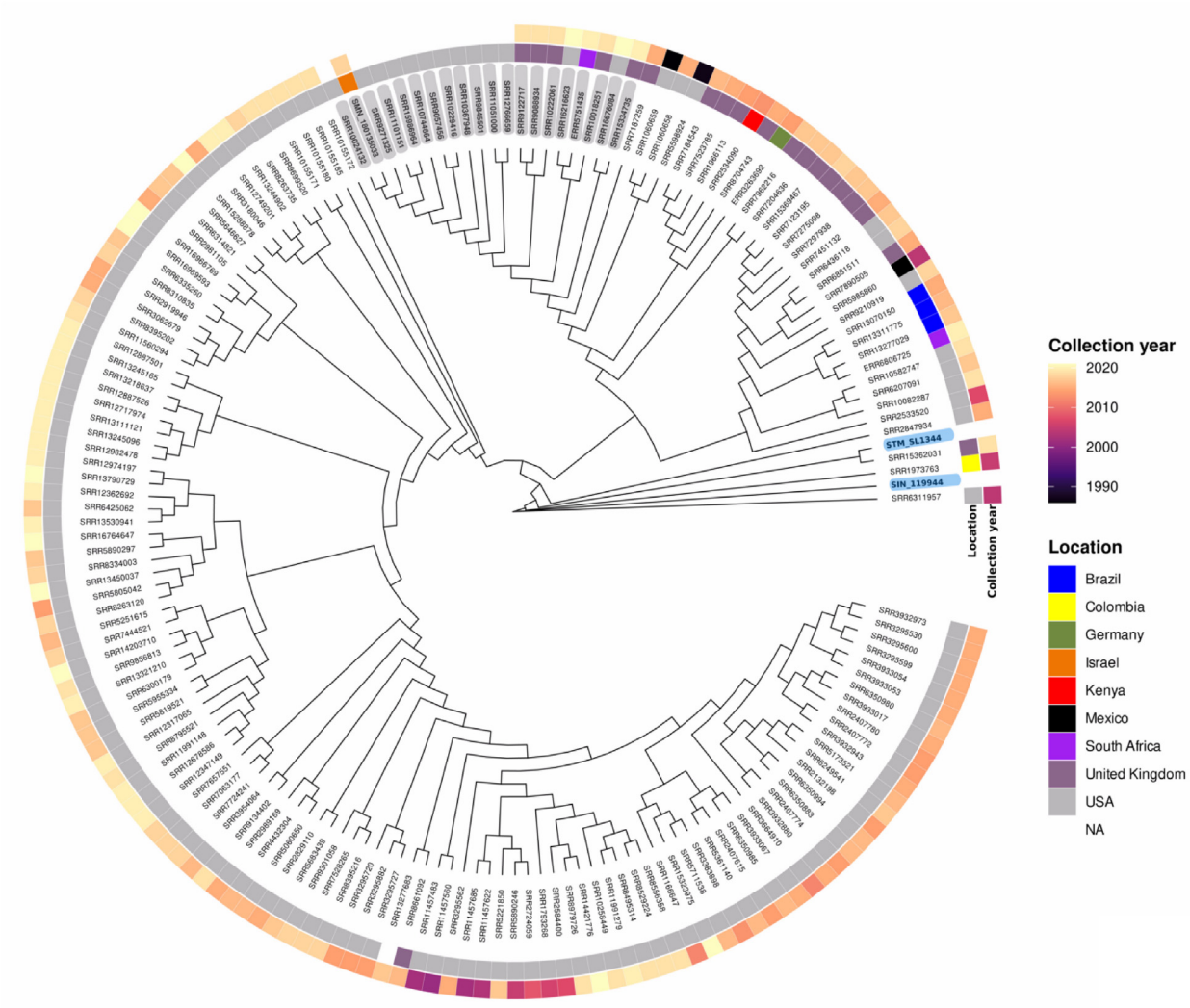

Fig. 4. Genetic relationship between global *S.* Muenchen resistant strains. A phylogenetic tree of the *S.* Muenchen 180135033 genome (including pESI) and the assembled SRA sequences of 168 *S.* Muenchen strains that harbor similar resistance profile was created using PhaME. The genomes of *S.* Typhimurium SL1344 and *S.* Infantis 119944 were included as out groups (highlighted by blue shading). The year of isolation is shown by the outer color ring and the country of origin is indicated by the internal ring. *S.* Muenchen pESI-positive strains are highlighted in bold and grey shading.

including *aadA1*, *sul1*, *tetRA* and *dfrA1*, conferring resistance to streptomycin and spectinomycin, sulfonamide, tetracycline, and trimethoprim, respectively. In addition, it carries several virulence-associated gene clusters expected to contribute to its pathogenicity, host-specificity and environmental tolerance, including two chaperon-usher fimbria clusters (Ipf and Klf [24]), the mercury resistance operon, and the potent iron acquisition system, yersiniabactin [8] (Fig. 3B).

Distribution of pESI-positive S. Muenchen strains outside of Israel

Using the NCBI's Isolate Browser tool we were able to identify 168 *S.* Muenchen genomes with Sequence Read Archive (SRA) data that carry at least one AMR gene found in pESI (Supplementary Material Table S3). Out of which, 24 genomes encoded the four signature AMR genes *sul1*, *tetA*, *dfrA1*, and *aadA1*. Magic-BLAST tool was used to search for the presence of the *ybt*, yersiniabactin locus (26,912 bp) presets in pESI. Interestingly, 19 out of these 24 genomes were found to contain the complete *ybt* cluster. Guided and de-novo assembly of these 19 SRA data sets, further confirmed the presence of pESI. Fig. 3B highlights the high sequence similarity between the pESI plasmid found in the Israeli *S.* Muenchen isolate 180135033 and pESI from clinical *S.* Muenchen strains that were isolated in USA during 2021 (BioSample SAMN22081945; SRA number SRR16216623), and during May 2019 in Gauteng, South Africa, from a human stool sample (BioSample SAMEA8598451; SRA number ERR5751435).

Next, PhaME [28] was used to analyze the phylogenetic relationship between *S.* Muenchen 180135033 (including pESI) and the 168 global *S.* Muenchen SRA sequences. This analysis showed that the Israeli strain and the 19 pESI-positive *S.* Muenchen isolates clustered together in the same clade (Fig. 4). These results indicate that genetically related *S.* Muenchen clinical isolates that carry pESI have been isolated recently in other countries beside Israel, including South Africa, UK, and USA.

pESI is self-transferable into other Salmonella serovars at varying frequencies

Given the finding that pESI has been acquired independently by two different *S. enterica* serovars, we next sought to investigate the host specificity of pESI by determining its ability to conjugate into *E. coli* and 11 prevalent *Salmonella* serovars. Conjugation experiment using *S.* Infantis 119944 strain as a donor to *E. coli* (strain ORN172), and 11 prominent *Salmonella* serovars (Muenchen, Typhimurium, Enteritidis, Infantis, Virchow, Kentucky, Bredeney, Anatum, Afula, Mbandaka, and Give) as recipients, showed variable conjugation frequency that ranges from zero to 8×10^{-6}. Comparison of the pESI conjugation frequency to *S.* Muenchen and the other serovars showed low conjugation frequencies ranged between zero to 9×10^{-7} in multiple strains of *S.* Afula, *S.* Anatum, *S.* Kentucky and *S.* Typhimurium, while significantly higher conjugation rates were observed in strains of *S.* Enteritidis, Give, Infantis, Mbandaka, and Virchow (Fig. 5). These results indicate that not all

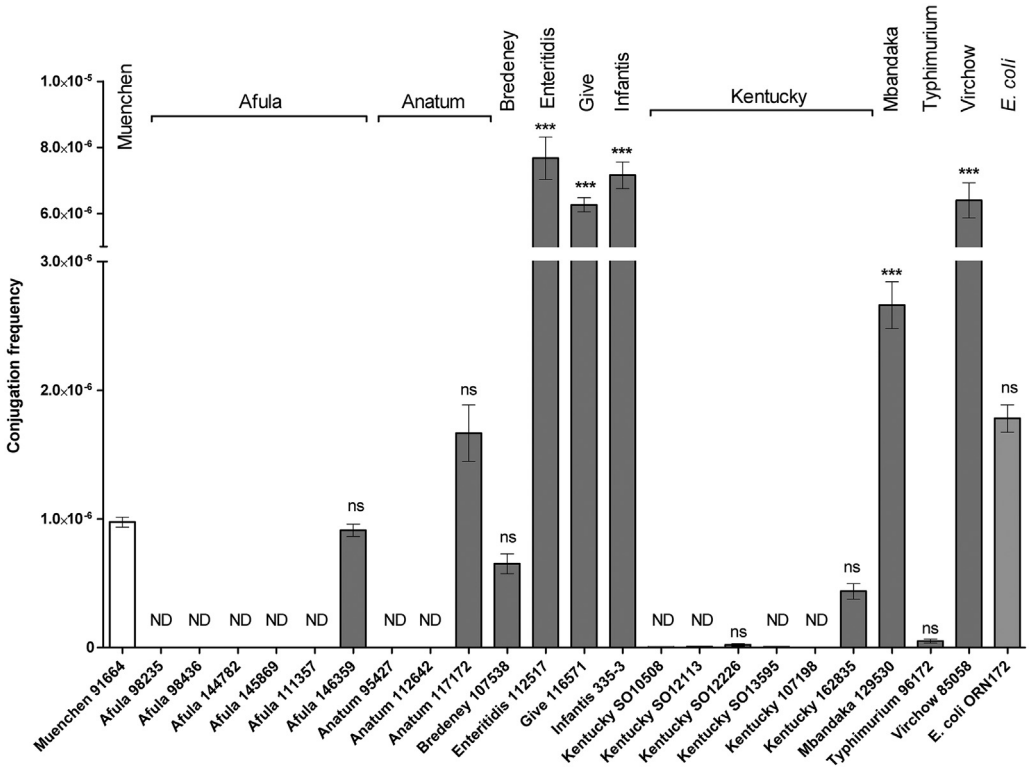

Fig. 5. pESI can be self-transferred to different *Salmonella* strains at varying frequencies. Mating experiments between *S.* Infantis 119944 that carries pESI used as the donor strain and 23 *Salmonella* isolates from 11 serovars (Typhimurium, Enteritidis, Infantis, Virchow, Kentucky, Bredeney, Muenchen, Anatum, Afula, Mbandaka, and Give) that were used as the recipient strains were conducted. Conjugation frequency was determined by the ratio between the number of transconjugant CFUs and the number of donor CFUs. The bars show the mean and the standard error of the mean (SEM) of three independent biological repeats. One-way ANOVA with Bonferroni's Multiple Comparison Test was used to calculate the statistical significance of the differences between the pESI conjugation frequency to *S.* Muenchen and the other strains. ND, not detected; ns, not significant; ***, $P < 0.0001$.

Salmonella strains have the same probability to acquire pESI and that pESI may have a different affinity (host-specificity) to certain serovars or even strains.

Discussion

Here we report a nation-wide dissemination of an MDR, pESI-positive *S*. Muenchen strain, firstly detected in Israel during 2018. The emergent *S*. Muenchen strain harbors a megaplasmid that is identical to the pESI plasmid of *S*. Infantis. The fact that the emergence of two independent *Salmonella* populations occurred within one decade (*S*. Infantis in 2008, and *S*. Muenchen in 2018), following horizontal acquisition of pESI is intriguing and reinforces the notion that pESI is an epidemic plasmid. Considering the genetic cargo of pESI that was previously shown to enhance resistance, stress tolerance and virulence [8,20,24], and its ability to conjugate at varying frequencies to prevalent *Salmonella* serovars, we suggest that pESI acquisition contributes to the fitness and resilience of its bacterial host [8,25] and could facilitate the emergence and dissemination of new *Salmonella* serovars.

High sequence similarity between the emergent Israeli *S*. Muenchen strain and the genomes of pESI-positive *S*. Muenchen isolates that were identified in USA, UK, and South Africa suggests a common origin, however, at this stage, the source and the transmission pathways of S. Muenchen pESI-positive strains outside of Israel are unknown. Based on the global previous experience gained with *S*. Infantis, it is possible that emergent MDR *S*. Muenchen strains that harbors pESI or pESI-like plasmids will be spreading in the future in other regions of the world.

The strengths of this study lie within its multidisciplinary nature that includes analysis of long-term national-wide epidemiological data, molecular biology, bacterial genetics, next generation sequencing and bioinformatics approaches. These allowed us to describe the clonal emergence of a new MDR *S*. Muenchen strain in Israel and identify the mechanism likely contributed to this reoccurring phenomenon.

One limitation of this study is the absence of very recent epidemiological data from the year 2021 that have not been published yet by the national Public Health Laboratories. Additional limitation is the rely on Sequence Read Archive data of *S*. Muenchen genomes that were deposited in publically available databanks for identification of similar strains circulating outside from Israel. It is highly possible that additional pESI-positive *S*. Muenchen strains, which their genome sequence was not determined or was not deposited in NCBI are currently distrubuted in additional countries.

Hence, improved WGS-based surveillance, international and timely data sharing, and alertness towards MDR *S*. Muenchen isolates in poultry and clinical samples are required to control a potentially global emergence of this epidemic strain.

Transparency declaration

The authors have declared that no competing interests exist. The work at the Gal-Mor laboratory was supported by grant numbers: I-41-416.6-2018 from the German-Israeli Foundation for Scientific Research and Development (GIF); A128055 from the Research Cooperation Lower Saxony – Israel (The Volkswagen Foundation); and 2616/18 from the joint ISF-Broad Institute program. The funders had no role in study design, data collection, and interpretation, or the decision to submit the work for publication.

Authors' contributions

Emiliano Cohen: Investigation, Methodology, Formal Analysis, Software, Data curation, Visualization, Writing – Original Draft. **Or Kriger**: Methodology, Investigation, Formal Analysis, Resources, Writing – Original Draft, Data curation, **Sharon Amit**: Resources, Supervision, Conceptualization, Project Administration, **Maya Davidovich**: Investigation, Formal Analysis, **Galia Rahav**: Conceptualization, Supervision, Project Administration, **Ohad Gal-Mor**: Conceptualization, Supervision, Funding acquisition, Writing – Original Draft, Writing – Review & Editing; Visualization, Project Administration.

Acknowledgements

We thank Dr. Assaf Rokney from the Central Laboratories, Ministry of Health and Dr. Udi Elnekave from the Hebrew University of Jerusalem for fruitful discussions.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2022.05.029.

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