Bacterial adaptation to venom in snakes and arachnida.

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Abstract

Animal venoms are considered sterile sources of antimicrobial compounds with strong membrane disrupting activity against multi-drug resistant bacteria. However, venomous bite wound infections are common in developing nations. Investigating the envenomation organ and venom microbiota of five snake and two spider species, we observe venom community structures that depend on the host venomous animal species, and evidence recovery of viable microorganisms from black-necked spitting cobra (*Naja nigricollis*) and Indian ornamental

tarantula (*Poecilotheria regalis*) venoms. Among the bacterial isolates recovered from *N. nigricollis* we identify two venom-resistant, novel sequence types of *Enterococcus faecalis* whose genomes feature 16 virulence genes indicating infectious potential, and an additional 45 genes, nearly half of which improve bacterial membrane integrity. Our findings challenge the dogma of venom sterility and indicate an increased primary infection risk in the clinical management of venomous animal bite wounds.

Importance

Notwithstanding their 3-5% mortality, the 2.7 million envenomation-related injuries occurring annually predominantly across Africa, Asia, and Latin America, are also major causes of morbidity. Venom toxin-damaged tissue will develop infections in some 75% of envenomation victims, with *E. faecalis* being a common culprit of disease; however, such infections are generally considered to be independent of envenomation. Here we evidence venom microbiota across snakes and arachnida, and report on the convergent evolution mechanisms that can facilitate adaptation to black necked cobra venom in two independent *E. faecalis* strains. Therefore, since inoculation with viable and virulent gene-harbouring bacteria can occur during envenomation, acute infection risk management following envenomation is warranted, particularly for immunocompromised and malnourished victims in resource-limited settings.

Main Text:

The rise of multi-drug resistant (MDR) bacterial infections suggests the end of the antibiotic golden era might be approaching fast. Discovery of novel antimicrobials is therefore an urgent

priority of exceptional socioeconomic value. Crude preparations of animal venoms exhibit strong antibiotic potencies, including against clinical MDR bacterial isolates such as *Mycobacterium tuberculosis* (1). With antimicrobial properties described for crotalid (pit viper) venom as early as 1948 (2), relevant compounds have been isolated from most animal venoms, including those of spiders, scorpions, and insects, as well as aquatic species. Examples include phospholipase-A2 enzymes, L-amino acid oxidases, cathelicidins, C-type lectins, and hydrophobic/cationic peptides, as well as venom toxin domains (3), which may act by physically disrupting bacterial cell membranes through pore formation (4, 5). Accordingly, venomous animal bite or sting (envenomation) wound infections are considered rare (6) and are attributed to secondary infection (7). Yet over three quarters of snake bite victims may develop mono- or polymicrobial envenomation wound infections, characterized by *Bacteroides*, *Morganella, Proteus*, *and Enterococcus* (8, 9) – bacterial taxa commonly found in the gut. Indeed, *Enterococcus faecalis* and *Morganella morganii* have been independently reported as the most common Gram positive and Gram negative envenomation wound infections across several countries (8–10). Historically associated with the oral snake microbiota (11), these bacteria are thought to originate from prey faeces (12) persisting in the snake oral cavity (10) with a diversity similar to that of the snake gut (13). Yet no 'fixed' oral microbiota were observed in early systematic studies, beyond a seasonal variation of diversity (14). Curiously, non-venomous snake mouths were reportedly more sterile than those of venomous snakes (14), a counterintuitive finding independently reproduced elsewhere (10). More recently, the oral microbiota of the non-venomous Burmese python (*Python bivittatus*) has also been reported to be native and not derived from prey guts (15).

As venom glands are connected to the tip of envenomation apparatus via a persistently open duct which is continuously exposed to the environment (16), envenomation apparatus could be

compared to clinical catheterisation assemblies: a transcutaneous needle resting on a non-sterile environment, connected to a continually open duct, leading to a liquid-containing vessel. Such devices develop biofilms within a few days, making weekly catheter replacement necessary (17). Unlike the high flow rates of catheters, however, envenomation apparatus normally ejects venom only sporadically. Captive snakes are often fed weekly and can fast for months whereas large arachnids are fed typically monthly. Wild animals may also undergo hibernation for several months when venom expulsion frequency can be assumed to be zero. Collectively, these conditions offer opportunities for microbes to colonise venom across its concentration gradient from the animal mouth to the venom gland, potentially driving the evolution of resistance against venom antimicrobials.

The aims of this study were to evaluate whether the microbiota of venom differs from that of the envenomating apparatus, whether microbes can survive in venom, and, if so, what genetic adaptations facilitate their survival in such extreme microenvironments. Using culture and culture-free methods we examine this hypothesis in snakes and spiders, and then employ antimicrobial susceptibility testing, whole genome sequencing analysis, and comparative genomics to identify and characterise venom tolerance and infectivity potential in representative novel *E. faecalis* isolates from captive *Naja nigricollis* (black-necked spitting cobra) venom.

Results

The snake venom microbiota vary on account of host species and not on account of the oral microbiota.

Applying established culture-free methods (18) on commercially available *Bothrops atrox* venom (*fer-de-lance*; Viperidae) and a venom sample from a captive *Bitis arietans* (African puff adder), we first optimised microbial DNA extraction for this unusual biological matrix **(Fig. S1)**. Given animal availability, behavioural, and sampling limitations such as the limited and inconsistent venom yields of scorpions, we next focused on five snake and two spider species (**Table 1**). We collected a swab (O) of the oral cavity (snakes) or fang surface (spiders) and two consecutive envenomation samples (E1 and E2), expecting the second venom sample to have fewer contaminants from bacterial plugs possibly forming on the envenomation apparatus. In agreement with previous reports (11, 14), principal coordinate analysis and unsupervised clustering (**Fig. S2**) failed to discriminate the swab microbiota by host species, suggesting the common diets and, most likely, water sources in captivity had the biggest impact.

This led us to hypothesise that captive animals would feature more closely related microbiota in their venoms compared to commercial or wild samples. We therefore compared the venom microbiota of all snakes using the same approach (**Fig. 1**). High Shannon-Wiener indices indicated considerable diversity in snake venom microbiota, however, surprisingly closer relationships were observed between *B. arietans* and other Viperidae, despite samples spanning captive and wild animals; an exception was *B. atrox* venom, which was characterized principally by *Gammaproteobacteria.* Focusing on *B. arietans* also failed to cluster samples by origin (**Fig. S3**), despite the unknown providence of the commercially available lyophilised sample, and the disparate locations across South Africa where wild *B. arietans* samples were collected (**Fig. 1D**); among the latter, the air-drying method used for venom preservation could have been expected to substantially compromise the microbiota signature. In contrast, *N. nigricollis* microbiota largely formed a distinct cluster (**Fig. 1**) characterized by *bacteroidia* (*bacteroidaceae*), a taxon less

common among Viperidae. This could reflect anatomical differences in elapid (cobra) fang location at the front of the mouth compared to the sheathed nature of the longer, hinged viperid fangs, whose tips rest at the back of the oral cavity. In contrast, spider species did not seem to influence venom microbiota consistency and exhibited lower biodiversity (**Fig. S4)**. These results likely reflected vertebrate/invertebrate anatomical differences and the limited venom yield from invertebrates (<1-30 μL) vs snakes (100-1,000 μL).

A fifth of the *N. nigricollis* venom microbiota is distinct from that of fangs.

Encouraged by the distinctive bacterial taxonomies in *N. nigricollis* venom, the availability of animals under controlled conditions, and the paired nature of the fang swab and envenomation samples, we delved deeper into this dataset. The fang microbiota appeared to form a distinct cluster to that of venom microbiota (**Fig. 2A, B)** suggesting the venom gland might be a distinct ecological niche (**Fig. 2C**). We therefore asked if any bacterial taxa were unique to subsets of these samples. Operational taxonomic unit (OTU) incidence analysis within each animal (**Fig. 2D**) suggested some 60% of OTUs were shared between corresponding venoms and fangs. Yet, importantly, up to 20% of these appeared to be unique to venom, and some 15% were unique to the fang (**Fig. 2E**), indicating an OTU continuum between the two microenvironments, with unique taxa in each site. Common sample types also featured a majority of common taxa, and OTUs unique to each site in each animal **(Fig. 2F**). However, taxa unique to each sample type (O, E1 or E2) were rarely found across all animals. These results suggested that although the microbiota between each snake fang and venom was largely common, venom contained distinct organisms.

The venom microbiota in snakes and spiders is viable.

After identifying all cultivable and non-cultivable bacterial species in swabs and venoms, we next proceeded to examine if cultivable aerobes could be recovered from these samples, as an indication of adaptation to venom. Testing for microbial viability (**Table S1**) yielded less growth with swab samples. Where this was significant, it was not usually matched by similar growth from the corresponding venom samples, further suggesting that the venom bacteria were probably not mouth contaminants. Strikingly, substantial and consistent growth was encountered amongst *N. nigricollis* (**Fig. 3A)** and *P. regalis* (**Table S1**) samples on blood agar. Unexpectedly, neither the wild (air dried) nor the commercial (lyophilised) venom samples yielded any growth, although colonies were obtained in blood agar from the captive *B. arietans* fangs, underscoring the impact of venom handling on microbial viability, at least for aerobic bacteria.

Clinical biochemistry tests identified the multiple, punctate white colonies from *N. nigricollis* almost universally as *Staphylococcus* spp., albeit with assay confidence intervals (CI) below 50% (**Table S2**). In contrast, *Stenotrophomonas maltophila* (80.4% CI) was present in five out of six *P. regalis* (all animals positive) and two *Lasiodora parahybana* (salmon pink tarantula) venom samples, but not on any fang swabs. Perplexed by the *N. nigricollis* results we sequenced these isolates on the Ion Torrent PGM.

Viable bacteria in *N. nigricollis* venom are two new *E. faecalis* sequence types.

Resequencing against putative reference genomes (**Table S2**) demonstrated less than 6% base alignment across all isolates. Instead, BLASTn analysis of the largest *de novo* assembled contigs identified *E. faecalis* V583 as the closest likely relative (>80% base alignment, 51.2x coverage).

This microbe is considered of mammalian origin, and is usually found in soils, waters, and foodstuffs probably arising from mammalian gastrointestinal tracts via faeces. This was puzzling given the catalase positive biochemistry of the isolates vs. the generally accepted catalase negative nature of *E. faecalis* (19), but explained by confirming the *E. faecalis* V583 *katA* gene, a haem-dependent cytoplasmic catalase (20), amongst all isolates at 99% identity. Blinded multiple sequence alignment (MSA) further revealed two *katA* alleles: one among isolates from animals 1 and 2 (allele 1) vs. another found in animal 3 isolates (allele 2; **Fig. 3B)** varying by less than 20 single nucleotide polymorphisms to the V583 allele (**Fig. S5A**). Interestingly, these alleles grouped isolates according to the origin and joint housing histories of animal 3 (group A) vs animals 1 and 2 (group B).

To explore isolate relationships further we generated minimum spanning trees (MST; **Fig. 3C**) by multi-locus sequence typing (MLST; **Table 2**), including at core genome level (cgMLST; **Fig. 3D** and **Fig. S5B-D**) (21, 22). Comparisons to five complete *E. faecium* genomes succeeded only for the *gyd* (alelles 16, 19) and *adk* (allele 18) loci. In contrast, MLST succeeded for all *E. faecalis* loci (**Table 2**), reinforcing *katA* allele observations (**Fig. 3B**), and identifying two novel sequence types featuring two new alleles for *pstS* and *yqiL* (**Fig. S6**) as confirmed by Sanger sequencing. MLST also indicated closer relationships to the *E. faecalis* strains OG1RF (87.5% +/- 1.7 of OG1RF cgMLST targets), D32 (78.8% +/- 2.1), and DENG1 (77.4% +/- 1.5). Pairwise comparisons of the resulting custom cgMLST schema including 5041 loci found across all the *N. nigricollis*-derived isolates further reinforced isolate grouping (**Fig. 3C**), suggesting two independent *E. faecalis* strain acquisition events across these three animals.

Pangenomic and experimental evidence of *E. faecalis* isolate adaptation to venom

Expanding cgMLST by an additional 3060 loci found in some *E. faecalis* isolates (**Fig. S5D**) identified 290-831 allelic differences within each animal. Whilst 80.9% of alleles varied between the two nearest neighbour isolates from the two strains, venom isolates from group B animals were divergent by 7.15 – 10.3% to their oral isolate counterparts, indicating that genomic changes were occurring with increased frequency, possibly in response to selective pressure applied by the venom. Given the well-described plasticity of the *E. faecalis* genome (OG1RF: 2.74 Mb vs V583: 3.36 Mb), we next examined mobile element divergence as the potential source of this adaptation. Detecting the pTEF2 gene *repA-2* (plasmid initiator protein) (**Table S3**) suggested only plasmid fragments were found in these genomes. As pTEF2 is one of three *E. faecalis* V583 plasmids associated to vancomycin resistance (23), we next confirmed the presence of fragments from the other pTEF plasmids in patterns consistent with the isolate groupings (**Table S4**), and some sequences absent from group A secondary envenomation isolates (**Fig. 4A)**. As many of the genomic elements with high (>95%) sequence identity to these plasmids were known, highly mobile sequences (e.g. the *E. faecalis* Bac41 bacteriocin locus) (24), these results suggested their participation in the genomic divergence of *E. faecalis* within each animal. Moreover, prompted by the detection of bacteriocin 41, a search for additional bacteriocins revealed a distinct cadre of genes among the two isolate groups. Thus, group A featured a class II bacteriocin, MR10A, MR10B, and enterolysin A, whilst group B shared carnocyclin A (bacteriocin IId), sonorensin, and a putative bacteriocin ABC transporter. Such antimicrobial peptide findings indicated that these *E. faecalis* strains had additional competitive advantages against other oral/fang bacteria, that could contribute to their positive selection among the microbiota attempting colonisation of *N. nigricollis* venom.

To ascertain if genes unique to these strains were responsible for adaptation to venom, we undertook closer examinations of their draft genome assemblies. Group A isolates shared ~2.9 Mb genomes (2,772-2,836 genes), with group B genomes varying from 3.04 to 3.24 Mb in size (3,128 to 3,282 genes; **Table S3**). Including the strains OG1RF and V583 derived an *E. faecalis* pangenome consisting of 5,130 genes, 1,977 of which formed a core genome that retained venom isolate groupings distinct to OG1RF and V583 (**Fig. 4B)**. After annotation, 235 genes were identified as specific to group A, and 321 genes as specific to group B. Most interestingly, among a set of 45 genes unique to both group groups, Uniprot functional annotation indicated known functions for 46.7% of these (**Table S5**), with a subset of 15 genes (35.7%) associated to cell wall/membrane integrity, and an additional 4 genes (9.53%) associated to pathogenic foreign protein and toxin defence. Significantly, pathway analysis via DAVID using *B. subtilis* orthologues identified 8-fold enrichment ($p=0.018$) in the two-component system pathway, specifically genes responsive to cationic antimicrobial peptides, cell wall active antimicrobials, and bacitracin efflux – mechanisms which are collectively compensatory to the well-established antimicrobial activity of venoms.

To ascertain the origin of these isolates and expand our search for proteins with functions opposing venom antimicrobial activity, we extended comparisons to 723 additional *E. faecalis* genome sequences in GenBank. This increased the pangenome by 5-fold (26,412 genes) with only 342 genes highly conserved among all 734 strains. A maximum-likelihood tree from the core genome alignment separated the venom-derived strains into different groups (**Fig. 4C**): placed within distinct subclades relative to isolates of diverse origin globally, including animal, environmental and human sources, there was no obvious geographical or other link to the two venom-derived strains (**Fig. S7**). Genome comparisons (70% identity threshold) in this wider

context identified 42 genes unique to group A and 97 genes unique to group B (**Table S6**): Among proteins with annotated functions, genes with similar functions were also observed in other *E. faecailis* strains (**Table S7-8**) such as S3_02356 encoding a colanic acid biosynthesis protein, functionally represented by gene ef95_02851 in strain 7330112-3. The relevance of such genes in adaptation or tolerance to venom will require further molecular characterisation in the future.

We next looked for genes whose functions could counteract *N. nigricollis* venom components such as phospholipase A2 (PLA2), L-amino oxidase, and three-finger peptides (3FTX) that facilitate disruption of membrane integrity in bacteria (25–29). Putative candidates included homologs to acyltransferase-acyl carrier protein synthetase (Aas) previously reported to protect bacterial cell envelope from Human PLA2 in Gram-negative bacteria (30), and homologs to cell wall (*dltA*) and cell membrane (*mprF*) polyanions previously associated with sensitivity to human PLA2 in *S. aureus* (31). Interestingly, the *mprF* gene appeared disrupted into two smaller proteins in one Group B *E. faecalis* isolate (V31_01061 and V31_01062; **Table S9**). 3 copies of sortase family proteins were also detected: Sortase A has been associated with the resistance to human PLA2 in *Streptococcus pyogenes* (32). A further 21 genes involved in oxidative stress response, encoding various antioxidative enzymes such as superoxide dismutase, catalase, and glutathione metabolism which would counteract reactive oxygen species produced by venom L-amino acid oxidase activity (25) were also found; these proteins also contribute to the virulence of *E. faecalis* strains (**Table S9**; (33–35)).

Taken together, genome analysis of these *E. faecalis* strains indicated they were well-equipped to survive the stress imposed by *N. nigricollis* venom, a tenet supported experimentally, since V583 growth was dose-dependently inhibited at a minimum concentration of 11.7 mg/mL (95% CI:

9.36-14.6 mg/mL) and non-inhibitory concentration of 2.78 ng/ml (95% CI: 2.21-3.50 mg/mL) of filter-sterilised, freeze-dried *N. nigricollis* venom in brain heart infusion broth. By stark contrast, all the venom-derived strains exhibited <30% growth inhibition even at 50 mg/mL freeze-dried venom concentrations i.e. ~4x lower than the 208 mg/ml concentration of fresh *N. nigricollis* venom, resulting in ambiguous non-inhibitory concentration ranges of 25.2-44.0 mg/ml (no CI's calculable), with the group A strain V33 exhibiting no susceptibility to the inhibitory effects of venom **(Fig. S8).**

Projecting primary infection clinical risk from venom-tolerant *E. faecalis* isolates.

Since these viable *E. faecalis* strains could potentially infect envenomation wounds, we next looked for known resistance determinants that might facilitate opportunistic primary infection. None of these strains had any acquired resistance genes to any antimicrobial classes (**Table S10**), although all isolates featured *lsaA* (**Fig. S9**), an intrinsic streptogramin resistance gene (36): Since horizontally acquired genes are largely responsible for resistance to vancomycin, aminoglycosides, macrolides, and tetracycline in *Enterococcus* (37), these strains were considered susceptible to drugs in each of these drug classes. In addition, the absence of known resistance-associated mutations in *gyrA* (DNA gyrase), *parC* (DNA topoisomerase), and the 23S rRNA genes also increased the likelihood of susceptibility to oxazolidinones and fluoroquinolones. Thus, several available antimicrobials would likely be effective in treating infections caused by these strains of *E. faecalis*. However, a gene related to macrolide export (*macB5*) was detected in the venom-tolerant strains (**Table S5**) and several virulence genes (38, 39) were also identified. Among all isolates, these included conjugative plasmid transfer pheromone genes associated with virulence (*cCF10, cOB1, cpd, cad, camE*), endocarditis and

biofilm formation-associated pilus subunit genes (*ebpA, ebpB, ebpC, srt, pil*), a biofilm on plastic operon gene (*bop*), quorum sensing (*fsrA, fsrB, fsrC*) and virulence-associated Fsr locus genes such as gelatinase (*gelE*) and serine protease E (*sprE*), and other important virulence genes such as hyaluronidases A and B (*hylA, hylB*), thiol peroxidase for oxidative stress resistance (*tpx),* adhesin to collagen of *E. faecalis* (*ace),* endocarditis specific antigen/*E. faecalis* antigen A (*efaA*), and enterococcal leucine-rich protein A (*ElrA*) that prevents macrophage chemotaxis to *E. faecalis* and endocytosis. Furthermore, all six group B isolates had the aggregation substance (*agg*) gene that enhances macrophage escape by suppressing respiratory burst. Therefore, despite the absence of cytolysin and glycopeptide resistance genes (e.g. *cylA, cylB, cylM,* and *vanA, vanC,* respectively), both sequence types appeared well-equipped to establish infections in human patients following envenomation.

Discussion

In contrast to the generally held view that venoms are both antimicrobial (1–4) and sterile (6, 7, 40) despite contrasting reports since the 1940's (41), we show that microorganisms can viably colonise venoms of both vertebrates and invertebrates. Moreover, significant adaptation appears to be necessary in genes that counter the mechanisms of action of known, venom-derived antimicrobial peptides and enzymes (4, 5) to attain resistance to venom. Although we documented this in multiple isolates of two independently acquired *E. faecalis* strains, adaptation appeared to occur in parallel within each of the 3 black-necked cobras from whence the isolates were obtained. It is unclear to which extent this form of parallel convergent evolution extends beyond *N. nigricollis, Enterococcus* spp. or other antimicrobial resistance mechanisms, such as antibiotic resistance genes against last resort antibiotics found on mobile genetic elements (42,

43). This work therefore adds to the body of evidence (44) supporting further scrutiny of host-microbe interactions in the venomous microenvironment in understanding microbial adaptation mechanisms to extreme environmental challenges.

Identification of *E. faecalis* as the most prevalent culturable microbe across our European *N. nigricollis* venom samples strikingly reflects three independent clinical reports across Africa and Asia that this non-sporulating microbe is the most common Gram-positive infection cultured from infected envenomation wounds (8–10). Likewise, *E. faecalis* were the most common aerobic Gram-positive isolates in *N. naja* oral swabs (n=6) (45). Post-envenomation cellulitis and dermatitis, presumed bacterial in nature, was additionally observed in 25% of a 16-case series of *Steatoda nobilis* (false widow spider) envenomations in the UK and Ireland: one of these required intravenous penicillin and flucloxacilline treatment after hospital admission (46). *S. nobilis* chelicerae, were previously found to harbour 11 bacterial taxa and 22 separate bacterial species, including class 2 pathogens; 3 of these 22 species showing multi-drug resistance (47). Although explicit genomic evidence connecting venom microbes to envenomation infection remains elusive, in an experimental rabbit model of dermonecrosis (48) caused by *Loxosceles intermedia* (recluse spider) venom, *Clostridium perfringens* recovered from the spider fang and venom enhanced disease symptoms. *Stenotrophomonas*-like bacteria were also found to dominate cone snail venom microbiomes (49), indicating that microbial venom adaptation may extend well beyond snakes, spiders, scorpions, and snails. Furthermore, building upon the few instances of polymicrobial infection reported clinically (8–10), the reports on *L. intermedia* (48), *Conus* (49), and herein suggest that diverse microbes effectively co-colonise venom glands in host species-specific manner, and thus envenomation wounds. Envenomation wound management (40) should therefore extend beyond simply managing the severe tissue damage and

necrosis that might be caused by venomous bites, to include clinical microbiology on envenomation wounds upon presentation. This would be particularly relevant to individuals immunocompromised through disease or malnutrition, e.g. in developing nations where envenomation incidence rates are high, or to children on a venom/colony forming unit dose per body weight basis.

Yet common microbial diagnostic methods relevant to resource limited settings mistook *E. faecalis* for *Staphylococcus*, which could lead to unfavourable clinical decision making. Although many of the same antibiotics, including vancomycin and linezolid, would be considered for treatment of both staphylococci and enterococci, there are some potential differences. For instance, oxacillin is often employed as a first-line agent to treat *Staphylococcus* (50). This drug is not effective against enterococci, as the use of penicillins for *E. faecalis* infections would typically involve ampicillin, usually in combination with an aminoglycoside (51). In addition, cephalosporins such as cefotaxime are considered second-line therapies for coagulase-negative staphylococci such as *S. epidermidis* (52). However, enterococci are intrinsically resistant to this class of drugs, and their prevalence in the gut tends to increase in response to cephalosporin therapy (53). Thus, while the *E. faecalis* strains in this study did not have any known acquired resistance determinants, if they were to cause infections, ensuring their proper identification would be critical to issuing correct treatment and achieving positive clinical outcomes.

It is unclear at present how frequent misidentification events might be. At least one retrospective study reported higher incidence of *Staphylococcus* spp. in envenomation wounds (12), and Blaylock's seminal snake oral microbiota studies also reported *Proteus* and *Staphylococcus* (14): both relied on the same methods we used in this study that misidentified the pathogen. Our

results therefore further support use of PCR/sequencing methods as they become more relevant to resource limited settings (54), and suited to the point of need (55), in line with World Health Organisation ASSURED criteria. Understanding the sensitivity of these methods will be crucial in their reliable implementation in envenomation care. It is therefore noteworthy that despite the limited biomass levels in these samples, species-level OTU analysis on MG-RAST (56) correctly identified *E. faecalis* as one of the principle aerobic isolates in *N. nigricollis* venom. Thus, a simple phylogenetic or metagenomic approach, combined with local herpetogeography knowledge, could quickly and accurately inform clinical action regarding antivenom administration without relying on descriptions or capture of the offending animal, or unreliable antibody-based venom identification kits (57).

To conclude, we evidence that vertebrate and invertebrate animal venoms host diverse, viable microbiota, with isolates genetically adapted to venom antimicrobials of medical interest against MDR. These results challenge perceptions on the sterility of venom and absence of primary infection risk upon envenomation, pointing to modern nucleic acid technologies to better inform envenomation care and antibiotic use.

Materials and Methods

Animals and sampling

All samples analyzed in this study were provided by Venomtech Ltd., with the exception of freeze-dried *B. arietans* venom (Latoxan, Portes les Valence, France) and air-dried field collected samples of *B. arietans* venom collected in South Africa (Table 1). Briefly, captive animals were housed in 2m by 1m wooden, glass fronted vivaria with large hide, thermal gradient and water *ad-libitum*. All procedures for venom collection and swabbing were approved as unregulated under the

Animals (Scientific Procedures) Act 1976. Venom was collected by standard techniques; briefly snakes were restrained behind the head and presented to a collection vessel. Snakes freely bit into the vessel until envenomation was observed. Each snake was presented to two sterile collection vessels in succession, one for the first envenomation with potential fang plug, and the other for the second flow (labelled E1 and E2, respectively). While the snake was positioned over the second vessel, the oral cavity was swabbed with a sterile swab with individual collection tubes (invasive sterile swab with transport media, DeltaLab, VWR, Lutterworth, UK). The venom collection vessels were clear, sterile 125 mL polypropylene containers (ThermoFisher Scientific Ltd., Paisley, UK) covered by 2 x 9 cm^2 pieces of parafilm stretched to fit (ThermoFisher Scientific Ltd.). The collection vessel was secured to a bench during collection. After collection, aseptically dispensed aliquots were stored in individual 1 ml sterile, DNA free, polypropylene collection tubes (FluidX Ltd, Nether Alderley, UK), at -80^{o}C. Samples collected in the field were from wild puff adders sampled as part of a previous phylogeographic study (58). Venom samples were collected using a similar method to that described for captive animals, except that the entire venom sample was collected in a single collection vessel. Samples were lyophilised by storing <100 µl venom aliquots in a vacuum-sealed container that was half-filled with silica gel for 1-2 days at room temperature. Following drying, venom samples were stored in a refrigerator at 5°C. Snake venom composition and its constituent proteins have been shown to be remarkably resistant to alterations in storage conditions (59) and to degradation during long-term storage (60). We have further observed that our air dried, field collected samples for rattlesnake venoms show identical proteomic profiles as those obtained from other research groups using freeze dried venoms (61, 62). We thus reasoned that variation in storage conditions in this study would be unlikely to have substantially altered the basic properties of the venom substrate available for microbial growth.

Lasiodora parahybana and *Poecilotheria regalis* were housed in 5 and 8 L polypropylene boxes (Really Useful Products Ltd, Normanton, UK), respectively, with moist vermiculite (Peregrine Livefoods Ltd., Ongar, UK), plastic hide and 5 cm water bowl. Arachnids were anaesthetised with a rising concentration of carbon dioxide, the fangs were swabbed with a sterile swab which was then placed in an individual 1 mL sterile, DNA free, polypropylene collection tube (FluidX Ltd), and venom was subsequently collected from arachnids by electrical stimulation. All samples were stored at -80˚C. The same transport swabs (VWR) as those used for snakes were also used for invertebrate oral / fang swabbing. Samples were stored at 4˚C and cultured within 24 hours of collection.

Microbial culture

Aerobic microbial viability was determined by plating swabs or aliquoting 10 µl volumes of venom samples onto oxalated whole horse blood agar, MacConkey agar, or mannitol salt agar (ThermoFisher Scientific) plates and incubating at 30˚C for 72 hours. Biochemical isolate identification was undertaken using API® strips (20E, 20NE and Staph) interpreted via the APIWEB interface (BioMerieux, Basingstoke, UK). All isolates were stored on beads at -80˚C at the University of Westminster microbial isolate library. *N. nigricollis* sub-culture was performed by restoring cryogenically stored bacteria on lysogeny broth agar (ThermoFisher Scientific) grown for 48 hours at 30˚C, and single colony overnight culture in lysogeny broth (ThermoFisher Scientific) using aerated culture (300 rpm). Minimum inhibitory concentrations and non-inhibitory concentrations were determined by broth microdilution assays (63) in brain heart infusion media by measuring absorbance at OD_{600} on a Tecan Spark Cyto 96 plate reader (Tecan, Männedorf,

Switzerland) and computed in GraphPad Prizm v.9.2.0 according to Lambert and Pearson (64). All bacterial agar and broth materials were purchased from Formedium Ltd. (Norfolk, UK).

DNA extraction

Neat venom samples or samples diluted in 18 megaohm water previously confirmed as bacterial DNA free by 16S PCR were subjected to DNA extraction using TRIzol™, PureLink Genomic DNA kits or MagMAX Cell-Free DNA kits (ThermoFisher Scientific) according to the manufacturer's instructions. For combined extraction of Gram positive and Gram negative bacteria from liquid samples, diluted samples were split in equal volumes and processed according to the manufacturer's Gram wall specific lysis protocols, with lysates combined prior to DNA binding onto columns by simple admixture. DNA content was then analyzed by Nanodrop (ThermoFisher Scientific) spectrophotometry and purified material was stored at -80°C until further analysis.

16S phylogenetic library preparation and sequencing.

For short amplicon library preparation, the hypervariable V3 region of the 16S rDNA gene was amplified from 20 ng of DNA using the primers 5′-CCTACGGGAGGCAGCAG-3′ and 5′-ATTACCGCGGCTGCTGG-3′ (Integrated DNA Technologies BVBA, Leuven, Belgium),(18) 1U Platinum® PCR SuperMix High Fidelity (ThermoFisher Scientific) and 10 µM of primer-mix. The reaction mixes were incubated at 94 °C for 5 min followed by 30 cycles of 30 seconds at 94 °C, 30 seconds at 55 °C and 1 min at 72 °C and then final elongation at 72 °C for 10 min using a Techne Prime Thermal cycler (ColePalmer, Staffordshire, UK). PCR products (193 bp) were confirmed by 2% w/v agarose gel electrophoresis in TAE buffer (ThermoFisher Scientific).

NGS library preparation was carried out using the Ion Plus Fragment Library Kit according to the manufacturer's instructions (Rev. 3, ThermoFisher Scientific), except that reactions were reduced to 1/5th volumes. Pooled libraries were diluted to ~26 pM for templating on the Ion OneTouch 2 system (ThermoFisher Scientific) using the Ion PGM Template OT2 200 v2 kit according to the manufacturer's instructions (Rev. B, ThermoFisher Scientific). Templated samples were sequenced on the Ion Torrent Personal Genome Machine (PGM; ThermoFisher Scientific) system on a single 318 Ion Chip (ThermoFisher Scientific) using the Ion PGM™ 200 Sequencing kit according to the manufacturer's instructions (Rev G., ThermoFisher Scientific).

Whole genome sequencing.

DNA extracted from cultured isolates was mechanically sheared using the Covaris S220 Focused-ultrasonicator (Covaris, Brighton, UK). NGS libraries were generated using the NEBNext Fast DNA Library Prep Set for Ion Torrent (New England Biolabs, Hitchin, UK). Pooled samples were size selected with the LabChip XT (LabChip XT DNA 300 Assay Kit; PerkinElmer, Seer Green, UK) and diluted to 26 pM for templating with the Ion OneTouch 2 system using the Ion PGM Template OT2 200 kit. Templated samples were sequenced on the Ion PGM using the Ion PGM™ Sequencing 200 v2 reagent Kit (ThermoFisher Scientific) and Ion 318™ v2 Ion Chip (ThermoFisher Scientific).

Bioinformatic analyses

Raw Ion Torrent sequencing data reads were quality controlled and demultiplexed using the standard Ion Server v. 4.0 pipeline (ThermoFisher Scientific). Referenced and *de novo* assemblies were carried out using TMAP v.4.0 and the SPAdes plugin in the Ion Server. Phylogenetic data

analyses were carried out after independent data deposition and curation on the MG-RAST v.3.0 pipeline (56) (project IDs MGP5177 and MGP5617) which uses a BLAST approach and the EBI-METAGENOMICS v.1 (project ID ERP004004) pipeline (65) which uses a Hidden Markov Model approach. Raw 16S sequencing reads were deposited in the European Nucleotide Archive (PRJEB4693). Quality control for both resources included length and quality filtering followed by a dereplication step where sequences with identical 50 nucleotides in 5' positions were clustered together. MG-RAST taxonomy annotation involved RNA identification using VSearch, and assignments using a custom database generated by 90% identity clustering of SILVA, GreenGenes and RDP prokaryotic databases. EBI-METAGENOMICS identified rRNA using Hidden Markov Models present in the RDP databases and assigned taxonomy using Qiime and the GreenGenes prokaryotic database.

For post-processing analyses, the EBI-curated dataset was analyzed using MEGAN v.5.5.3 (66). Classical multi-locus sequence typing (http://efaecalis.mlst.net/) and cgMLST (21, 22) were carried out using Ridom SeqSphere+ v.4.0 running on a 2 core, 10 GB RAM, 500 GB hard disk Biolinux v.8.0 installation on a VirtualBox virtual machine instance on a 16GB RAM, 1TB hard disk Apple iMac. Extended cgMLST analysis to include partially detected loci, excluded loci annotated as 'failed' due to sequencing error suggesting genuine *E. faecalis* genomic divergence occurring within each animal. Plasmid detection was carried out using the PlasmidFinder v.1.3 server (67), followed by NCBI BLASTn analysis to detect shorter fragments, e.g. the same 398 nt fragment of *repA-2* in animal 3 isolates (<40% of the full-length gene) at 90.1% identity to the plasmid-borne reference sequence. Single gene comparisons and multiple sequence analyses were carried out using TCoffe and MView on the EMBL-EBI server, with base conservation visualized

by BoxShade v.3.3.1 on mobyle.pasteur.fr. Genome-level plasmid coverage analyses were carried out by NCBI BLASTn and comparisons were visualized using Circos v.0.69-4.

The sequencing reads were assembled using SPAdes v.3.9.0 (68), and the draft assemblies were annotated using Prokka (69) before NCBI deposition (BioProject No. PRJNA415175). The genome sequences of *E. faecalis* strains V583, OG1RF (Accession numbers NC_004668.1 and NC_017316.1, respectively) and 723 other *E. faecalis* strains were obtained from GenBank and were re-annotated using Prokka to have an equivalence of annotation for comparative analyses. The genomes were compared using the program Roary with a protein similarity threshold of 70% (70, 71). A maximum-likelihood tree was constructed from the core genomic alignment using IQ-Tree (72) with 100,000 ultra-fast bootstraps and 100,000 SH-aLRT tests. The tree was visualized using Interactive Tree Of Life (iTOL) (73).

To identify acquired resistance genes, nucleotide BLAST analysis was performed on the ResFinder (74) and NCBI (https://www.ncbi.nlm.nih.gov/pathogens/) resistance gene databases using cutoffs of 50% length and 85% identity to known resistance determinants. Additional BLAST analysis was performed to identify single nucleotide polymorphisms in the quinolone resistance determining region (QRDR) of *gyrA* and *parC* (75). Additional mutational analysis was performed on region V of the 23S rRNA-encoding genes (76). Virulence genes were identified using a combination of VirulenceFinder 2.0 (https://cge.cbs.dtu.dk/services/VirulenceFinder/; default parameters of 60% length and 90% identity to known *Enterococcus* virulence genes (77–79)) and manual BLAST with an evalue cut-off of 10^{-5}. Bacteriocins and ribosomally synthesized and post-translationally modified peptides were mined using BAGEL4 (https://bagel4.molgenrug.nl (80)).

BLASTP was performed in Ensembl Bacteria (release 38), against the *E. faecalis* V583 and *E. faecalis* (GCA_000763645) to obtain further geneID's from significant matches. *Bacillus subtilis* orthologue gene ID's were collated as this species is the closest relative to *E. faecalis* (VetBact.org) with the most comprehensive genome annotation required for gene onotolgy and KEGG pathway analysis. From the 42 genes unique to venom isolates, useable *B. subtilis* GeneID's were obtained for 20, of which 18 of these successfully converted to ENTREZ Gene ID's using the functional annotation tool (DAVID Bioinformatics resource 6.8 (81, 82)), selecting *B. subtilis* as the background species.

References and Notes:

1. Xie JP, Yue J, Xiong YL, Wang WY, Yu SQ, Wang HH. 2003. In vitro activities of small peptides from snake venom against clinical isolates of drug-resistant Mycobacterium tuberculosis. International Journal of Antimicrobial Agents 22:172–174.
2. Glaser HSR. 1948. Bactericidal Activity of Crotalus Venom in Vitro. Copeia 1948:245.
3. Samy R, Gopalakrishnakone P, Satyanarayanajois S, Stiles B, Chow V. 2013. Snake Venom Proteins and Peptides as Novel Antibiotics Against Microbial Infections. Current Proteomics 10:10–28.
4. Stocker JF, Traynor JR. 1986. The action of various venoms on Escherichia coli. Journal of Applied Bacteriology 61:383–388.

5. Perumal Samy R, Stiles BG, Franco OL, Sethi G, Lim LHK. 2017. Animal venoms as antimicrobial agents. Biochemical Pharmacology 134:127–138.
6. Talan DA, Citron DM, Overturf GD, Singer B, Froman P, Goldstein EJ. 1991. Antibacterial activity of crotalid venoms against oral snake flora and other clinical bacteria. J Infect Dis 164:195–8.
7. Powers DW. 2005. Stings and bites: what to do about envenomation injuries. Emerg Med Serv 34:67, 69–75, quiz 99.
8. Wagener M, Naidoo M, Aldous C. 2017. Wound infection secondary to snakebite. The South African Medical Journal 107:315–319.
9. Mao YC, Liu PY, Hung DZ, Lai WC, Huang ST, Hung YM, Yang CC. 2016. Bacteriology of Naja atra snakebite wound and its implications for antibiotic therapy. American Journal of Tropical Medicine and Hygiene 94:1129–1135.
10. Lam KK, Crow P, Ng KHL, Shek KC, Fung HT, Ades G, Grioni A, Tan KS, Yip KT, Lung DC, Que TL, Lam TSK, Simpson ID, Tsui KL, Kam CW. 2011. A cross-sectional survey of snake oral bacterial flora from Hong Kong, SAR, China. Emergency Medicine Journal 28:107–114.
11. Jorge MT, de Mendonça JS, Ribeiro LA, da Silva ML, Kusano EJ, Cordeiro CL. 1990. [Bacterial flora of the oral cavity, fangs and venom of Bothrops jararaca: possible source of infection at the site of bite]. Revista do Instituto de Medicina Tropical de Sao Paulo 32:6–10.
12. Garg A, Sujatha S, Garg J, Acharya NS, Parija SC. 2009. Wound infections secondary to snakebite. Journal of Infection in Developing Countries 3:221–223.

13. Iqbal J, Sagheer M, Tabassum N, Siddiqui R, Khan NA. 2014. Culturable Aerobic and Facultative Anaerobic Intestinal Bacterial Flora of Black Cobra (Naja naja karachiensis) in Southern Pakistan. ISRN Vet Sci 2014:878479.
14. Blaylock RS. 2001. Normal oral bacterial flora from some southern African snakes. Onderstepoort J Vet Res 68:175–82.
15. Costello EK, Gordon JI, Secor SM, Knight R. 2010. Postprandial remodeling of the gut microbiota in Burmese pythons. The ISME Journal 4:1375–1385.
16. Trim SA, Trim CM. 2013. Venom: the sharp end of pain therapeutics. Br J Pain 7:179–88.
17. Loveday HP, Wilson JA, Pratt RJ, Golsorkhi M, Tingle A, Bak A, Browne J, Prieto J, Wilcox M. 2014. Epic3: National evidence-based guidelines for preventing healthcare-associated infections in nhs hospitals in england. Journal of Hospital Infection 86.
18. Muyzer G, de Waal EC, Uitterlinden AG. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol1993/03/01. 59:695–700.
19. Fisher K, Phillips C. 2009. The ecology, epidemiology and virulence of Enterococcus. Microbiology (N Y) 155:1749–1757.
20. Frankenberg L, Brugna M, Hederstedt L. 2002. Enterococcus faecalis heme-dependent catalase. J Bacteriol 184:6351–6.
21. Mellmann A, Harmsen D, Cummings CA, Zentz EB, Leopold SR, Rico A, Prior K, Szczepanowski R, Ji Y, Zhang W, McLaughlin SF, Henkhaus JK, Leopold B, Bielaszewska M, Prager R, Brzoska PM, Moore RL, Guenther S, Rothberg JM, Karch H. 2011. Prospective Genomic Characterization of the German Enterohemorrhagic

Escherichia coli O104:H4 Outbreak by Rapid Next Generation Sequencing Technology. PLoS ONE 6:e22751.

22. de Been M, Pinholt M, Top J, Bletz S, Mellmann A, van Schaik W, Brouwer E, Rogers M, Kraat Y, Bonten M, Corander J, Westh H, Harmsen D, Willems RJL. 2015. Core Genome Multilocus Sequence Typing Scheme for High-Resolution Typing of Enterococcus faecium. Journal of Clinical Microbiology 53:3788–3797.

23. Paulsen IT, Banerjei L, Myers GSA, Nelson KE, Seshadri R, Read TD, Fouts DE, Eisen JA, Gill SR, Heidelberg JF, Tettelin H, Dodson RJ, Umayam L, Brinkac L, Beanan M, Daugherty S, DeBoy RT, Durkin S, Kolonay J, Madupu R, Nelson W, Vamathevan J, Tran B, Upton J, Hansen T, Shetty J, Khouri H, Utterback T, Radune D, Ketchum KA, Dougherty BA, Fraser CM. 2003. Role of Mobile DNA in the Evolution of Vancomycin-Resistant Enterococcus faecalis. Science (1979) 299:2071–2074.

24. Kurushima J, Ike Y, Tomita H. 2016. Partial Diversity Generates Effector Immunity Specificity of the Bac41-Like Bacteriocins of Enterococcus faecalis Clinical Strains. J Bacteriol 198:2379–90.

25. Samel M, Tõnismägi K, Rönnholm G, Vija H, Siigur J, Kalkkinen N, Siigur E. 2008. l-Amino acid oxidase from Naja naja oxiana venom. Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology 149:572–580.

26. Ferraz CR, Arrahman A, Xie C, Casewell NR, Lewis RJ, Kool J, Cardoso FC. 2019. Multifunctional toxins in snake venoms and therapeutic implications: From pain to hemorrhage and necrosis. Frontiers in Ecology and Evolution. Frontiers Media S.A. https://doi.org/10.3389/fevo.2019.00218.

27. Williams HF, Hayter P, Ravishankar D, Baines A, Layfield HJ, Croucher L, Wark C, Bicknell AB, Trim S, Vaiyapuri S. 2018. Impact of naja nigricollis venom on the production of methaemoglobin. Toxins (Basel) 10.

28. Rheubert JL, Meyer MF, Strobel RM, Pasternak MA, Charvat RA. 2020. Predicting antibacterial activity from snake venom proteomes. PLoS ONE 15.

29. Bocian A, Hus KK. 2020. Antibacterial properties of snake venom components. Chemical Papers. Springer https://doi.org/10.1007/s11696-019-00939-y.

30. Lin Y, Bogdanov M, Lu S, Guan Z, Margolin W, Weiss J, Zheng L. 2018. The phospholipid-repair system LplT/Aas in Gram-negative bacteria protects the bacterial membrane envelope from host phospholipase A2 attack. Journal of Biological Chemistry 293:3386–3398.

31. Koprivnjak T, Peschel A, Gelb MH, Liang NS, Weiss JP. 2002. Role of charge properties of bacterial envelope in bactericidal action of human Group IIA phospholipase A2 against Staphylococcus aureus. Journal of Biological Chemistry 277:47636–47644.

32. Movert E, Wu Y, Lambeau G, Touqui L, Areschoug T. 2011. A Novel Bacterial Resistance Mechanism against Human Group IIA-Secreted Phospholipase A 2 : Role of Streptococcus pyogenes Sortase A . The Journal of Immunology 187:6437–6446.

33. Patel MP, Marcinkeviciene J, Blanchard JS. 2006. Enterococcus faecalis glutathione reductase: purification, characterization and expression under normal and hyperbaric O2 conditions. FEMS Microbiology Letters 166:155–163.

34. Szemes T, Vlkova B, Minarik G, Tothova L, Drahovska H, Turna J, Celec P. 2010. On the origin of reactive oxygen species and antioxidative mechanisms in Enterococcus faecalis. Redox Report. Redox Rep https://doi.org/10.1179/135100010X12826446921581.

35. Imlay JA. 2013. The molecular mechanisms and physiological consequences of oxidative stress: Lessons from a model bacterium. Nature Reviews Microbiology. Nat Rev Microbiol https://doi.org/10.1038/nrmicro3032.

36. Singh K v, Weinstock GM, Murray BE. 2002. An Enterococcus faecalis ABC homologue (Lsa) is required for the resistance of this species to clindamycin and quinupristin-dalfopristin. Antimicrob Agents Chemother 46:1845–50.

37. Hollenbeck BL, Rice LB. 2012. Intrinsic and acquired resistance mechanisms in enterococcus. Virulence 3:421–569.

38. Nami Y, Haghshenas B, Haghshenas M, Khosroushahi AY. 2015. Antimicrobial activity and the presence of virulence factors and bacteriocin structural genes in Enterococcus faecium CM33 isolated from ewe colostrum. Frontiers in Microbiology 6:782.

39. Hashem YA, Abdelrahman KA, Aziz RK. 2021. Phenotype–Genotype Correlations and Distribution of Key Virulence Factors in Enterococcus faecalis Isolated from Patients with Urinary Tract Infections. Infection and Drug Resistance 14:1713.

40. Palappallil DS. 2015. Pattern of Use of Antibiotics Following Snake Bite in a Tertiary Care Hospital. J Clin Diagn Res 9:OC05-9.

41. Pasricha CL, Abedin Z. 1941. The Sterility of Snake Venom Solutions. Ind Med Gaz 76:276–277.

42. Liu Y-Y, Wang Y, Walsh TR, Yi L-X, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu L-F, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu J-H, Shen J. 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. The Lancet Infectious Diseases 16:161–168.

43. McGann P, Snesrud E, Maybank R, Corey B, Ong AC, Clifford R, Hinkle M, Whitman T, Lesho E, Schaecher KE. 2016. Escherichia coli Harboring *mcr-1* and *bla* $_{\text{CTX-M}}$ on a Novel IncF Plasmid: First Report of *mcr-1* in the United States. Antimicrobial Agents and Chemotherapy 60:4420–4421.

44. Ul-Hasan S, Rodríguez-Román E, Reitzel AM, Adams RMM, Herzig V, Nobile CJ, Saviola AJ, Trim SA, Stiers EE, Moschos SA, Keiser CN, Petras D, Moran Y, Colston TJ. 2019. The emerging field of venom-microbiomics for exploring venom as a microenvironment, and the corresponding Initiative for Venom Associated Microbes and Parasites (iVAMP). Toxicon X 4.

45. Panda SK, Padhi L, Sahoo G. 2018. Oral bacterial flora of Indian cobra (Naja naja) and their antibiotic susceptibilities. Heliyon 4:1008.

46. JP D, A V, DT O, A F, R S, MM D. 2021. Bites by the noble false widow spider Steatoda nobilis can induce Latrodectus-like symptoms and vector-borne bacterial infections with implications for public health: a case series. Clin Toxicol (Phila) https://doi.org/10.1080/15563650.2021.1928165.

47. JP D, NA K, CL A, P B, J M, S A, V O, MM D, A B. 2020. Synanthropic spiders, including the global invasive noble false widow Steatoda nobilis, are reservoirs for medically important and antibiotic resistant bacteria. Sci Rep 10.

48. Monteiro CLB, Rubel R, Cogo LL, Mangili OC, Gremski W, Veiga SS. 2002. Isolation and identification of Clostridium perfringens in the venom and fangs of Loxosceles intermedia (brown spider): Enhancement of the dermonecrotic lesion in loxoscelism. Toxicon 40:409–418.

49. Torres JP, Tianero MD, Robes JMD, Kwan JC, Biggs JS, Concepcion GP, Olivera BM, Haygood MG, Schmidt EW. 2017. Stenotrophomonas-Like Bacteria Are Widespread Symbionts in Cone Snail Venom Ducts. Appl Environ Microbiol 83:AEM.01418-17.
50. Andrews JM, Boswell FJ, Wise R. 2000. Establishing MIC breakpoints for coagulase-negative Staphylococci to oxacillin. J Antimicrob Chemother 45:259–61.
51. Arias CA, Contreras GA, Murray BE. 2010. Management of Multi-Drug Resistant Enterococcal Infections. Clinical Microbiology and Infection 16:555–62.
52. Aldridge KE. Cefotaxime in the treatment of staphylococcal infections. Comparison of in vitro and in vivo studies. Diagn Microbiol Infect Dis 22:195–201.
53. de Vries-Hospers HG, Tonk RH, van der Waaij D. 1991. Effect of intramuscular ceftriaxone on aerobic oral and faecal flora of 11 healthy volunteers. Scand J Infect Dis 23:625–33.
54. Quick J, Loman NJ, Duraffour S, Simpson JT, Severi E, Cowley L, Bore JA, Koundouno R, Dudas G, Mikhail A, Ouédraogo N, Afrough B, Bah A, Baum JHJ, Becker-Ziaja B, Boettcher JP, Cabeza-Cabrerizo M, Camino-Sánchez Á, Carter LL, Doerrbecker J, Enkirch T, Dorival IG-, Hetzelt N, Hinzmann J, Holm T, Kafetzopoulou LE, Koropogui M, Kosgey A, Kuisma E, Logue CH, Mazzarelli A, Meisel S, Mertens M, Michel J, Ngabo D, Nitzsche K, Pallasch E, Patrono LV, Portmann J, Repits JG, Rickett NY, Sachse A, Singethan K, Vitoriano I, Yemanaberhan RL, Zekeng EG, Racine T, Bello A, Sall AA, Faye O, Faye O, Magassouba N, Williams C v., Amburgey V, Winona L, Davis E, Gerlach J, Washington F, Monteil V, Jourdain M, Bererd M, Camara A, Somlare H, Camara A, Gerard M, Bado G, Baillet B, Delaune D, Nebie KY, Diarra A, Savane Y, Pallawo RB, Gutierrez GJ, Milhano N, Roger I, Williams CJ, Yattara F, Lewandowski K,

Taylor J, Rachwal P, J. Turner D, Pollakis G, Hiscox JA, Matthews DA, Shea MKO, Johnston AMcD, Wilson D, Hutley E, Smit E, di Caro A, Wölfel R, Stoecker K, Fleischmann E, Gabriel M, Weller SA, Koivogui L, Diallo B, Keïta S, Rambaut A, Formenty P, Günther S, Carroll MW. 2016. Real-time, portable genome sequencing for Ebola surveillance. Nature 530:228–232.

55. Shah K, Bentley E, Tyler A, Richards KSR, Wright E, Easterbrook L, Lee D, Cleaver C, Usher L, Burton JE, Pitman JK, Bruce CB, Edge D, Lee M, Nazareth N, Norwood DA, Moschos SA. 2017. Field-deployable, quantitative, rapid identification of active Ebola virus infection in unprocessed blood. Chem Sci 8:7780–7797.

56. Wilke A, Bischof J, Gerlach W, Glass E, Harrison T, Keegan KP, Paczian T, Trimble WL, Bagchi S, Grama A, Chaterji S, Meyer F. 2016. The MG-RAST metagenomics database and portal in 2015. Nucleic Acids Research 44:D590–D594.

57. Johnston CI, Ryan NM, Page CB, Buckley NA, Brown SG, O'Leary MA, Isbister GK. 2017. The Australian Snakebite Project, 2005-2015 (ASP-20). Med J Aust 207:119–125.

58. Barlow A, Baker K, Hendry CR, Peppin L, Phelps T, Tolley KA, Wüster CE, Wüster W. 2013. Phylogeography of the widespread African puff adder (Bitis arietans) reveals multiple Pleistocene refugia in southern Africa. Mol Ecol 22:1134–57.

59. Munekiyo SM, Mackessy SP. 1998. Effects of Temperature and Storage Conditions on the Electrophoretic, Toxic and Enzymatic Stability of Venom Components. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 119:119–127.

60. Hatakeyama DM, de Morais-Zani K, Serino-Silva C, Grego KF, Sant'Anna SS, Fernandes W, Aniz PAEA, Torquato RJS, Tanaka AS, Sanz L, Calvete JJ, Tanaka-Azevedo AM. 2018. Examination of biochemical and biological activities of Bothrops jararaca

(Serpentes: Viperidae; Wied-Neuwied 1824) snake venom after up to 54 years of storage. Toxicon 141:34–42.

61. Massey DJ, Calvete JJ, Sánchez EE, Sanz L, Richards K, Curtis R, Boesen K. 2012. Venom variability and envenoming severity outcomes of the Crotalus scutulatus scutulatus (Mojave rattlesnake) from Southern Arizona. Journal of Proteomics 75:2576–2587.

62. Zancolli G, Calvete JJ, Cardwell MD, Greene HW, Hayes WK, Hegarty MJ, Herrmann HW, Holycross AT, Lannutti DI, Mulley JF, Sanz L, Travis ZD, Whorley JR, Wüster CE, Wüster W. 2019. When one phenotype is not enough: divergent evolutionary trajectories govern venom variation in a widespread rattlesnake species. Proceedings of the Royal Society B 286.

63. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically.

64. Lambert RJW, Pearson J. 2000. Susceptibility testing: accurate and reproducible minimum inhibitory concentration (MIC) and non-inhibitory concentration (NIC) values. J Appl Microbiol 88:784–790.

65. Hunter S, Corbett M, Denise H, Fraser M, Gonzalez-Beltran A, Hunter C, Jones P, Leinonen R, McAnulla C, Maguire E, Maslen J, Mitchell A, Nuka G, Oisel A, Pesseat S, Radhakrishnan R, Rocca-Serra P, Scheremetjew M, Sterk P, Vaughan D, Cochrane G, Field D, Sansone S-A. 2014. EBI metagenomics—a new resource for the analysis and archiving of metagenomic data. Nucleic Acids Research 42:D600–D606.

66. Huson DH, Beier S, Flade I, Górska A, El-Hadidi M, Mitra S, Ruscheweyh H-J, Tappu R. 2016. MEGAN Community Edition - Interactive Exploration and Analysis of Large-Scale Microbiome Sequencing Data. PLoS Comput Biol 12:e1004957.
67. Carattoli A, Zankari E, García-Fernández A, Voldby Larsen M, Lund O, Villa L, Møller Aarestrup F, Hasman H. 2014. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother 58:3895–903.
68. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, Lesin VM, Nikolenko SI, Pham S, Prjibelski AD, Pyshkin A v., Sirotkin A v., Vyahhi N, Tesler G, Alekseyev MA, Pevzner PA. 2012. SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. Journal of Computational Biology 19:455–477.
69. Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069.
70. Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MTG, Fookes M, Falush D, Keane JA, Parkhill J. 2015. Roary: rapid large-scale prokaryote pan genome analysis. Bioinformatics 31:3691–3693.
71. Tange O. Gnu parallel-the command-line power tool. usenix.org.
72. Nguyen L-T, Schmidt HA, von Haeseler A, Minh BQ. 2015. IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. Molecular Biology and Evolution 32:268–274.
73. Letunic I, Bork P. 2016. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. Nucleic Acids Research 44:W242–W245.

74. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen M v. 2012. Identification of acquired antimicrobial resistance genes. Journal of Antimicrobial Chemotherapy 67:2640–2644.

75. Kanematsu E, Deguchi T, Yasuda M, Kawamura T, Nishino Y, Kawada Y. 1998. Alterations in the GyrA subunit of DNA gyrase and the ParC subunit of DNA topoisomerase IV associated with quinolone resistance in Enterococcus faecalis. Antimicrob Agents Chemother 42:433–5.

76. Marshall SH, Donskey CJ, Hutton-Thomas R, Salata RA, Rice LB. 2002. Gene dosage and linezolid resistance in Enterococcus faecium and Enterococcus faecalis. Antimicrobial Agents and Chemotherapy 46:3334–3336.

77. Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL. 2009. BLAST+: architecture and applications. BMC Bioinformatics 10.

78. Tetzschner AMM, Johnson JR, Johnston BD, Lund O, Scheutz F. 2020. In Silico genotyping of Escherichia coli isolates for extraintestinal virulence genes by use of whole-genome sequencing data. Journal of Clinical Microbiology 58.

79. Joensen KG, Scheutz F, Lund O, Hasman H, Kaas RS, Nielsen EM, Aarestrup FM. 2014. Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic Escherichia coli. J Clin Microbiol 52:1501–1510.

80. van Heel AJ, de Jong A, Song C, Viel JH, Kok J, Kuipers OP. 2018. BAGEL4: a user-friendly web server to thoroughly mine RiPPs and bacteriocins. Nucleic Acids Res 46:W278–W281.

81. Huang DW, Sherman BT, Lempicki RA. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature Protocols 4:44–57.

82. Huang DW, Sherman BT, Lempicki RA. 2009. Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Research 37:1–13.

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experiments together with ST. SD performed the MIC/NIC curve assays. All authors contributed equally to the overall interpretation of the dataset and manuscript preparation; **Competing interests:** Authors declare no competing interests.

Data availability: Phylogenetic data are deposited on MG-RAST (project IDs MGP5177 and MGP5617) and the EBI-METAGENOMICS servers (project ID ERP004004). Raw 16S sequencing reads were deposited in the European Nucleotide Archive (PRJEB4693). Annotated draft *E. faecalis* genome assemblies are deposited on NCBI (BioProject No. PRJNA415175).

Supplementary Materials:

Figures S1-S8

Tables S1-S10

Figure S1: 16S Ribosomal RNA gene PCR output and phylogenetic differences on account of venom collection and extraction methods. The choice of extraction method (phenol-chloroform-based: Trizol; column based; magnetic bead based) impacts significantly on the recovery and amplification of bacterial DNA from lyophilized *B. atrox* venom (A). This bacterial DNA is not an artefact of lyophilisation process contamination as detection is maintained in aseptically collected, flash-frozen *Bitis arietans* venom, nor is it an artefact of diluent contamination by 18 MΩ water confirmed 16S free by PCR; however, >10x dilution of venom is necessary for PCR to progress (B). The yield of bacterial DNA is a function of upstream cell

lysis methods selectivity for Gram +ve or Gram –ve cell walls (C). The cell lysis and extraction methodology also directly impact upon microbial diversity profiles as determined by 16S rRNA phylogenetics for either lyophilised (D) or aseptically collected, flash-frozen venoms (E), , with combined use of cell wall-specific extraction methods yielding more balanced profiles.. +C: positive control; -C: negative control; -ve: method specific negative controls.

Figure S2: Comparison of the oral microbiomes of snakes and spiders suggests their oral microbiota is not host-species specific. UPGMA tree (A - C) and PCoA (D – F) analysis (Bray-Curtis indeces) of the oral microbiome diversity of snakes (A, D; individual species identified by independently coloured dots), spiders (B, E; *L. parahybana*: red dots, *P. regalis*: black dots), or vertebrate vs invertebrate animals (C, F; black vs red dots), as determined by 16S rRNA phylogenetic analysis at class level indicate no host species-specific relationships. Dots represent single captivity individuals, labelled with short species name, enumerated for individual number and identified for the oral/fang (O) nature of the sample.

Figure S3: The origin of a *B. arietans* snake does not appear to influence the microbiome profile in the venom of each animal. *B. arietans* venom microbiome profiles do not present substantial differences on account of host geographical origin as determined by A) PCoA, B) UPGMA tree and C) class-level taxonomic profiling following 16S rRNA phylogenetic analysis. Dots in (A) and (B) represent individual animal data, are coloured and labelled by animal origin and number (red B1-B8: wild; blue L: lyophilised captivity (Latoxan); black B. are01-E1: flash-frozen captivity; Venomtech). Relative taxonomic diversity profiles in (C) are aligned to the UPGMA tree sample labels, with the Shannon-Wiener Index (SWI) of each sample indicated.

Sample B3 was removed from the analysis due to the ~100x lower read depth yield from this sample compared to all other *B. arietans* samples.

Figure S4: Spider venom microbiome profiles suggest closer relationships between consecutive envenomation samples within *P. regalis* individuals. Spider venom microbiomes were compared by A) PCoA, B) UPGMA tree and C) class-level taxonomic profiling following 16S rRNA phylogenetic analysis. Dots in (A) and (B) represent individual animal data, are colored/labelled by species and envenomation number (black and grey: *L. parahybana* envenomation 1 (E1) and 2 (E2) respectively; red and pink: *P. regalis* E1 and E2 respectively). Relative taxonomic diversity profiles in (C) are aligned to the UPGMA tree sample labels, with the Shannon-Wiener Index (SWI) of each sample indicated.

Fig. S5: MSA, MST and cgMLST define two novel *E. faecalis* sequence types isolated across *N. nigricollis* venom and oral cavities. Blinded MSA (A) of the *KatA* gene sequence across the nine *E. faecalis isolates* obtained from *N. nigricollis* oral swabs (O) and two consecutive envenomation samples (E1 and E2) from three independent animals (light blue (N.nig01), dark blue (N.nig02) and orange (N.nig03)) defines two alleles distinct to the V583 reference sequence (bottom lane). Base conservation is defined by similarity to the animal 1 and 2 *KatA* sequence using BoxShade v.3.3.1 on mobyle.pasteur.fr. Each pixel column represents a different nucleotide with orange and red columns indicating increasingly different nucleotides. Blinded MST analysis of these nine isolates against B) *E. faecalis* and *E. faecium* reference genomes (distance calculations based on *E. faecium* MLST), C) *E. faecalis* reference genomes with partial incidence locus data removed, and D) a custom cgMLST schema derived from *E.*

faecalis OG1RF, D32 and DENG1 including loci with partial data between all study isolates (8101 targets). Allelic differences in excess of 5% of the cgMLST schema are highlighted by '*'. Reference genomes: *E. faecalis*: V583, OG1RF, D32, DENG1, 29212, Symbioflor 1; *E. faecium*: T110; AUS0085, Aus0004, NRRL B-2354, DO.

Figure S6: Novel *pstS* and *yqiL* allele sequences obtained from *N. nigricollis* venom-derived *E. faecalis*. The sequences of the novel *pstS* (A) and *yqiL* (B) alleles found in a novel *E. faecalis* sequence type obtained from *N. nigricollis* venom (animal 3). Clustal omega alignments of the *yqiL* sequences (C) from *E. faecalis* isolates derived from animal 2 venom against *E. faecalis* *yqiL* allele 8 found in the orally-derived isolate. The alignment is focused to positions 301-319 of the 436 nt allele and single base pair indels are highlighted in red.

Figure S7: Source of *E. faecalis* strains with genomes closely related to venom-tolerant strains isolated from *N. nigricollis* venom. The genome record metadata available for the closest *E. faecalis* isolates related to (A) group A and (B) group B *N. nigricollis* venom isolates (subtrees extracted from the original 734 *E. faecalis* strain core genome tree) are depicted.

Figure S8: *N. nigricollis* venom-derived *E. faecalis* isolates resist the inhibitory effects of *N. nigricolis* lyophilised venom. The growth inhibitory effect of pooled, filter-sterilised, lyophilised *N. nigricollis* venom dissolved in brain heart infusion broth across 2-fold serial dilutions of 50 mg/ml was assessed for five *E. faecalis* isolates derived from *N. nigricollis* venom and the reference isolate V583 after 24 hr shaken incubation at 37^{o}C by turbidity assessment at 600 nm. Data representative of 3 independent replicate experiments.

Figure S9: Multiple sequence alignment of the *lsa(A)* gene reinforces the clustering of the *N. nigricollis*-derived *E. faecalis* isolates. Blinded MSA of the *lsa(A)* antibiotic resistance gene sequence across the nine *E. faecalis isolates* obtained from *N. nigricollis* oral swabs (O) and two consecutive envenomation samples (E1 and E2) from three independent animals (light blue (N.nig01), dark blue (N.nig02) and orange (N.nig03)) defines two alleles distinct to the TX0263 reference strain gene sequence (accession no. AY737526.1). Base conservation is defined by similarity to the animal 1 and 2 *lsa(A)* sequence using BoxShade v.3.3.1 on mobyle.pasteur.fr. Each pixel column represents a different nucleotide with orange and red columns indicating increasingly different nucleotides.

Figure 1: Snake venom microbiomes cluster on account of host species. Viperid venom microbiomes cluster separately from *N. nigricollis* with the exception of *B. atrox* as determined by A) PCoA, B) UPGMA tree and C) class-level taxonomic profiling following 16S rRNA phylogenetic analysis. Dots in (A) and (B) are coloured by species (red: *B. arietans*; black: *N. nigricollis*; light blue: *B. atrox*; orange: *C. atrox;* dark blue: *O. scutallatus*), represent data of captivity individuals, are labelled with short species name, enumerated for individual number and identified for the envenomation number (E1 or E2) of the sample. The 8 wild (red dots B1-B8) and the commercially sourced, lyophilised (red L dot) *B. arietans* sample are independently labelled. Sample B3 was removed from the analysis due to the ~100x lower read depth yield from this sample compared to all other *B. arietans* samples. Relative taxonomic diversity profiles in (C) are aligned to the UPGMA tree sample labels, with the Shannon-Wiener Index (SWI) of each sample indicated. The geographical origin of the wild *B. arietans* samples collected in South Africa are shown in D.

Figure 2: The intra- and inter-individual relationship of venom and oral microbiomes in *N. nigricollis*. Comparison of the oral and venom microbiomes in three *N. nigricollis* individuals by A) PCoA, B) UPGMA tree and C) class-level taxonomic profiling following 16S rRNA phylogenetic analysis indicates separate clustering of the microbiotae in the two microenvironments. D) Within animal incidence comparisons of operational taxonomic units (OTUs) suggest E) unique taxa exist within the oral but also the venom microenvironments. F) Between animal comparisons per niche (E1, E2, Oral) indicate most OTUs are shared but some OTUs are unique to each animal for each site. Dots in (A) and (B) represent individual *N. nigricollis* (N.nig) animal data and are coloured/labelled by sample type (black: oral; red: envenomation 1 (E1); blue: envenomation 2 (E2)). Relative taxonomic diversity profiles in (C) are aligned to the UPGMA tree sample labels, with the Shannon-Wiener Index (SWI) of each sample indicated. The 'venom' histogram in E) represents the sum OTU fraction found in the two envenomation samples per individual (+/- standard deviation).

Figure 3: Whole genome sequencing identifies viable bacteria in *N. nigricollis* venom as two, animal-specific, *E. faecalis* strains. (A) White punctate colonies were recovered in blood agar (upper panels) and MacConkey agar (lower panels) blinded cultures of individual oral swab (O) and two consecutive envenomation samples (E1 and E2) obtained from three captivity *N. nigricollis* snakes. N/D: none detected. (B) Blinded multiple sequence alignment (ClustalO followed by ClustalW phylogeny) of homologous sequences across the *de novo* assembled genomes against the *E. faecalis* V583 *KatA* gene (distance to V583 *KatA* indicated in V583 track) suggests two sequence groups reflecting the history and housing of the host animals. (C) Blinded MST construction based on the MLST of the *N. nigricollis*-derived isolates against nine *E. faecalis* reference genomes again separate samples into two distinct clusters that reflect the history and housing of the host animals. Partially available allele data are included in this analysis and allelic difference instances between nearest neighbours are annotated in white boxes. (D) Blinded complete genome MLST against a custom schema generated using three closely related *E. faecalis* reference genomes cluster these isolates by animal of origin (animals 1 (light blue), animal 2 (dark blue) and animal 3 (orange)). The host animal colour scheme depicted in D) is also used in B) and C).

Figure 4: Comparative genomics of mobile and core genomic chromosomal elements of venom-tolerant *E. faecalis* (A) Circos coverage plots of the vancomycin resistance-associated, V583 plasmids pTEF1, pTEF2 and pTEF3 in the *E. faecalis* isolates obtained from oral, envenomation 1 (E1) and envenomation 2 (E2) samples from three *N. nigricollis* individuals, reinforce the two sequence type groupings and highlight within animal variation (green arcs) indicative of sample-specific variation (lack of reads) across E2 samples in animals 1 and 2. The central plot for each plasmid and animal reflects GC content. All data is represented in 50 nt blocks. (B) Blinded maximum likelihood tree of the core genomic alignments for the 6 *N. nigricollis*- derived *E. faecalis* isolates against the V583 and OG1RF reference strains, with colour-coding refering to the origin of the isolates; light blue: animal 1; dark blue: animal 2, yellow: animal 3 (C) A maximum likelihood tree from concatenated nucleotide sequence alignment of 865 core genes (381,319 bp) from 734 genomes after removing the sites with gaps. Best-fit GTR+I+G4 substitution model was used with 100,00 ultra-fast bootstraps and SH-aLRT tests. The tree was re-rooted on the longest branch and branch lengths <0.001 were collapsed. The scale bar shows number of nucleotide substitutions per site. Branches in red, blue, purple and green show Group A, Group B, and clades containing strains V853 and OG1RF, respectively.

Table 1: Animals sampled for the presence of microbiomes in venom.

Common name	Scientific name	Short name	Origin	Preservation method	Number of animals.
Snakes					
Puff adder	*Bitis arietans*	*B.are*	Captivity	Flash-frozen	1
		L	Commercial	Lyophilised	1
		B1-B8	Wild	Air-dried	8
Black-necked cobra	*Naja nigricollis*	*N.nig*	Captivity	Flash-frozen	3
Fer-de-lance	*Bothrops atrox**	*B.atr*	Captivity	Flash-frozen	3
Western diamond rattlesnake	*Crotalus atrox**	*C.atr*	Captivity	Flash-frozen	2
Taipan	*Oxyuranus scutellatus**	*O.scu*	Captivity	Flash-frozen	2
Spiders					
Indian ornamental	*Poecilotheria regalis*	*P.reg*	Captivity	Flash-frozen	3
Salmon pink	*Lasiodora parahybana***	*L.par*	Captivity	Flash-frozen	5

* Venom produced by one animal only

** Yields ranged <1 - 30 µl.

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Table 2: Novel sequence types of *E. faecalis* recovered from fangs and venoms of *N. nigricollis.*

Animal no.	Sample		Locus						
	Isolate origin	Blinding code	*gdh*	*gyd*	*pstS*	*gki*	*aroE*	*xpt*	*yqiL*
1	O	S22	22	6	31	13	11	35	8
	E1	V36	22	6	31	13	11	35	8
	E2	V29	22	6	31	13	11	35	8
2	O	S17	22	6	31	13	11	35	8
	E1	V31	22	6	31	13	11	35	8*
	E2	V28	22	6	31	13	11	35	8**
3	O	S3	18	1	New	24	83	47	New
	E1	V33	18	1	New	24	83	47	New
	E2	V23	18	1	New	24	83	47	New

O: oral swab sample

E1: envenomation 1

E2: envenomation 2

*: single base pair deletion in NGS data not validated by Sanger sequencing

**: homopolymer single base extension not validated by Sanger sequencing