Livestock as possible reservoir of *Escherichia albertii* in Switzerland

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Nutztiere als mögliches Reservoir von *Escherichia albertii* in der Schweiz

Escherichia albertii ist ein neu auftretender zoonotischer lebensmittelassoziierter Krankheitserreger. Prävalenz, Verbreitung und mögliche Reservoire sind noch nicht eindeutig definiert. In dieser Studie haben wir das Vorkommen und die genomischen Eigenschaften von *E. albertii* bei Nutztieren in der Schweiz evaluiert. Zwischen Mai und August 2022 wurden insgesamt 515 Blinddarmproben von Schafen, Rindern, Kälbern und Mastschweinen während des Schlachtvorganges entnommen. Unter Verwendung einer *E. albertii*-spezifischen PCR, die auf das *Eacdt*-Gen abzielte, waren 23,7 % (51/215) der Schweine aus 24 verschiedenen Betrieben positiv. Eines (1 %) von 100 Kälbern zeigte ein positives PCR-Ergebnis, während alle Proben von Schafen und Rindern PCR-negativ waren. Acht *E. albertii*-Isolate konnten aus Schweineproben gewonnen und mittels Ganzgenomsequenzierung analysiert werden. Alle acht Isolate gehörten zu den MLST Sequenztypen ST2087 oder ST4619, ebenso wie die meisten Genome der 11 verfügbaren globalen Schweineisolate aus öffentlichen Datenbanken. Diese beiden Cluster teilten das Vorhandensein eines Virulenzplasmids, das die *sitABCD*- und *iuc*-Gene beherbergte. Zusammenfassend zeigen wir, dass Mastschweine ein *E. albertii*-Reservoir in der Schweiz darstellen und beschreiben spezifische Schweine-assoziierte Bakterienencluster.

Schlüsselwörter: *Escherichia albertii*, Schwein, Kälber, Rind, Schaf, Reservoir

Abstract

Escherichia albertii is an emerging zoonotic foodborne pathogen. Its prevalence, distribution, and reservoirs are not yet clearly defined. In this study, we evaluated the occurrence and genomic characteristics of *E. albertii* in livestock from Switzerland. A total of 515 caecal samples from sheep, cattle, calves, and fattening swine were collected between May 2022 and August 2022 at abattoir level. Using an *E. albertii*-specific PCR targeting the *Eacdt*-gene, 23,7 % (51/215) of swine from 24 different farms were positive. One (1 %) out of 100 calves showed a positive PCR result, while all samples from sheep and cattle were PCR negative. Eight *E. albertii* isolates could be recovered from swine samples and were analysed using whole-genome sequencing. All eight isolates belonged to ST2087 or a ST4619 group subclade, as did most genomes of the 11 available global swine isolates from public databases. These two clusters shared the presence of a virulence plasmid harboring the *sitABCD* and *iuc* genes. In summary, we demonstrate that fattening swine constitute an *E. albertii* reservoir in Switzerland and describe specific swine-associated lineages.

Keywords: *Escherichia albertii*, pig, swine, calves, cattle, sheep, reservoir

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Introduction

Escherichia albertii is a Gram-negative, nonmotile, non-spore-forming facultative anaerobe bacteria of the family of Enterobacterales.[22] This zoonotic pathogen has sporadically been associated with infectious diarrhea in humans.[1,4,10,16,24,31,36,37] Intimin, an *eae* gene-encoded outer membrane protein and type III secretion system effector, leading to attaching-and-effacing lesions in the intestinal epithelium, is one of the main virulence factors. Another *E. albertii*-specific virulence factor is the cytolethal distending toxin (*cdt*), which consists of three subunits (CdtA, CdtB, and CdtC). CdtB acts as a DNAse leading to DNA double-strand breaks and irreversible cell cycle arrest.[16] The role of this virulence factor is still not fully understood, but it has been associated with invasion and persistent colonisation.[11,12,39]

An overview of the importance and occurrence of *E. albertii* was recently published.[30] Difficulties in isolating this pathogen and misclassification usually as *E. coli* might be contributing to challenges in the full determination of this pathogen's reservoirs. However, wild birds and in some countries such as Japan and USA, poultry seem to play a major role as *E. albertii* reservoirs.[6,20,32,35,36] Raccoons are a further *E. albertii* reservoir in USA.[18,21] Although, a few studies have detected *E. albertii* in livestock or their meat, the significance of livestock as carriers of *E. albertii* is still inconclusive.[15,45]

In this study, we screened food-producing animals in Switzerland as potential carriers for *E. albertii*. Isolates were further characterised by whole genome sequencing.

Material and Methods

Sample collection

A total of 515 caecal samples from sheep (n = 100), cattle (n = 100), calves (n = 100), and fattening swine (n = 215) were collected between May 2022 and August 2022 at abattoir level. Caecum sampling side was chosen based on the pathogen monitoring (e.g. *Salmonella)* and the antibiotic resistance monitoring (e.g. ESBL Enterobacterales) in pig of the European Food Safety Authorithy (EFSA). The herds as well as the animals were randomly selected at different sampling days. Depending on the herd size, two to five animals were sampled per herd. Swab samples were aseptically collected from caecal contents by cutting through the caecal wall with sterile scissors. Post sampling each swab was placed in a sterile blender bag (Seward, Worthing, UK), placed into a cool box and transported to the laboratory. Microbiological examination was carried out within 2 to 4 h after sampling.

Bacterial Enrichment, Growth Conditions, and DNA Extraction

All swab samples were enriched and incubated overnight at 42°C in *Enterobacteriaceae* Enrichment (EE) broth (Becton Dickinson, Heidelberg, Germany). The next day, each enrichment culture was streaked onto sheep blood agar (DifcoTM Columbia Blood Agar Base EH; Becton Dickinson AG, Allschwil, Switzerland) and incubated again overnight at 42°C. After washing off the grown colonies with 2mL of 0,85 % NaCl, an aliquot (100 µL) of each suspension was combined with 200 µL of Gram-negative lysis buffer. This was heated at 60°C for 50 min followed by 99°C for 10 min. Centrifugation at 11,000rpm for 2 min then allowed the supernatant to be used as a template for PCR.

Screening for *Eacdt* genes

A PCR assay was used to screen for the presence of the *E. albertii*-specific cytolethal distending toxin (*Eacdt*)-gene.[30] The PCR was performed as previously described, using GoTaq® Green Master Mix (Promega, Madison, WI) as a minor modification.[17] With the primer set, EaCDTsp-F2 and EaCDTsp-R2, a 449 bp fragment of the *Eacdt*-gene was detected. *E. albertii* strain DSM 17528 served as positive control and purified water as negative control. The cycle conditions were 5 min at 94°C followed by 30 cycles of 30s at 94°C, 30s at 50°C, 40s at 72°C, and lastly 7 min at 72°C. The results were analysed using the Molecular Imager® Gel Doc™ XR system.

Isolation of *E. albertii*

In case of a positive *Eacdt*-PCR result, a loopful of the washed-off bacterial suspension was streaked onto Xylose-MacConkey-plates and incubated overnight at 42°C.[19] In contrast to *E. coli,* most *E. albertii* strains cannot ferment xylose, causing them to grow as colourless to grey-white colonies. These presumptive positive colonies were tested again for the presence of *Eacdt*. One confirmed *Eacdt* positive colony per sample was stored in 25 % glycerol at -80°C.

Whole-Genome Sequencing (WGS) and Genome Analysis

Strains were grown on sheep blood agar at 37° overnight. For short-read sequencing, DNA was isolated using the DNA Blood and Tissue Kit (Qiagen, Hombrechtikon, Switzerland). DNA libraries were prepared using the Nextera DNA Flex Sample Preparation Kit (Illumina, San Diego, CA, USA) and sequenced using the Illumina MiniSeq platform (Illumina, San Diego, CA, USA). Illumina read quality was assessed using FastQC 0.11.7.[2] Whole genome assemblies were generated with SPAdes 3.14.1 implemented in shovill 1.1.1 using default settings.[5,41]

For long-read sequencing, DNA was extracted using the MasterPure Complete DNA and RNA Purification Kit (Lucigen). Multiplex libraries were prepared using the SQK-LSK109 ligation sequencing kit with the EXP-NBD104 native barcoding expansion kit. Libraries were sequenced on a MinION Mk1B device using the FLO-MIN106 (R9) flow cell (Oxford Nanopore Technologies). Hybrid assemblies were produced with Unicycler 0,5.

Publicly available genomic data of *E. albertii* strains were obtained from NCBI Genbank or, when available, the NCBI Sequence Read Archive (SRA). Genomic data from 482 isolates described by Luo et al.[28], 8 additional swine isolates, and 12 isolates from wild birds recently recovered in Switzerland[6] were considered for inclusion. Read data were assembled using shovill 1.1.1. Assembly quality was assessed using QUAST 5.0.2 and CheckM 1.1.3.[14,40] Low-quality assemblies (n = 11) (N50 < 20 kb, CheckM completeness <96 %, CheckM contamination >3 %) were removed.

Multi-locus sequence types (STs) were determined using PubMLST (https://pubmlst.org/).[23] Acquired resistance genes were identified using AMRfinder 3.10.24 (default parameters).[9] Virulence genes were identified using ABRicate 1.0.1 (https://github.com/tseemann/abricate) in combination with the Virulence Factor Database set B (sequence coverage 50 %, identity 70 %).[26] The *eae*-gene was typed using ABRicate 1.0.1 (sequence coverage 70 %, identity 97 %) in combination with the *eae* database provided by Luo et al.[28] Subtypes of *cdtB* were identified using ABRicate 1.0.1 (sequence coverage 70 %, identity 97 %) with nucleotide sequences of accession numbers AAD10622 (*cdtB*-I), AAA18786 (*cdtB*-II), AAC45443 (*cdtB*-III/V), AAT92048 (*cdtB*-IV), and AST83_RS10865 (*cdtB*-VI) as references. *E. albertii* O- and H-antigen genotypes were determined by in silico PCR (https://github.com/egonozer/in_silico_pcr) in combination with primer pairs described by Ooka et al. and Nakae et al.[33,36] Only exact matches were considered. SNP distances were determined by read mapping using the CFSAN SNP Pipeline 2.2.1 for each ST group separately.[8] Core genome alignments were generated with parsnp v1.5.3.[43] Maximum-likelihood phylogenetic trees were constructed from the alignment using IQ-TREE v2.0.3 with the generalized time-reversible (GTR) model and gamma distribution with 100 bootstraps.[34]

Ethics statement

This study was performed in accordance with the Swiss Animal Welfare Act (SR 455) and is ethically accepted by the FSVO. All information was treated anonymously.

Results

Prevalence of *Eacdt* PCR positive samples

Out of 215 fattening swine caecal samples from 44 farms, 51 (23,7 %) were PCR positive for *Eacdt* (Table 1). The 51 swine originated from 24 different farms. One (1 %) out of 100 calf samples showed a positive PCR result, whereas all samples from sheep and cattle were PCR negative.

By streaking PCR positive colony suspensions onto Xylose-MacConkey plates, *E. albertii* isolates could be recovered from 8 out of 51 PCR positive swine samples, each originating from a distinct farm.

Genomic characterization of *E. albertii* isolates

All eight *E. albertii* whole-genome sequenced pig isolates belonged either to ST4619 (n = 3) or ST2087 (n = 5). High-resolution SNP analyses did not identify potential transmission clusters. Pairwise distances ranged from 15 to 111 SNPs among ST4619 isolates and from 34 to 1210 SNPs among ST2087 isolates, suggesting no recent epidemiological link between the isolates.

All isolates carried the *cdtB* subtype VI, with one isolate (KBSW201i) harbouring a second *cdt* operon (subtype I). Other genetic characteristics (*eae* subtypes, O- and H-antigen genotypes) were largely shared among isolates belonging to the same sequence type (Table 2). The three ST4916

Table 1: Number of *Eacdt*-positive caecal samples and number of recovered *E. albertii* isolates

	No. samples	No. (%) PCR positive samples	No. recovered *E. albertii* strains
Sheep	100	0	0
Fattening swine	215	51 (23,7 %)*	8+
Calves	100	1 (1 %)	0
Cattle	100	0	0

*Originating from 24 different farms; +originating from 8 different farms

Table 2: Characteristics of *E. albertii* isolates recovered from swine caecal samples

Isolate	MLST	*stx*	*eae* subtype *	*cdtB* subtype	O-antigen genotype	H-antigen genotype	Antimicrobial resistance genes	Accession number
KBSW16i	ST4619	–	nu	*cdtB*-VI	EAOg5	EAHg1	*aadA1, aph(3'')-Ib*	GCA_025600175.1
KBSW26i	ST4619	–	nu	*cdtB*-VI	EAOg5	EAHg1	*aadA1, aph(3'')-Ib*	GCA_025600145.1
KBSW214i	ST4619	–	nu	*cdtB*-VI	EAOg5	EAHg1	*aadA1, aph(3'')-Ib*	GCA_025600015.1
KBSW50i	ST2087	–	omicron	*cdtB*-VI	EAOg25	EAHg3	–	GCA_025600135.1
KBSW78i	ST2087	–	omicron	*cdtB*-VI	EAOg25	EAHg3	–	GCA_025600115.1
KBSW154i	ST2087	–	omicron	*cdtB*-VI	EAOg25	EAHg3	–	GCA_025600095.1
KBSW184i	ST2087	–	omicron	*cdtB*-VI	EAOg25	EAHg3	*aadA1, sat2*	GCA_025600055.1
KBSW201i	ST2087	–	omicron	*cdtB*-I, *cdtB*-VI	EAOg25	EAHg3	–	GCA_025600065.1

*according Luo et al.[28] ; ≥97 % sequence identity, 100 % sequence coverage

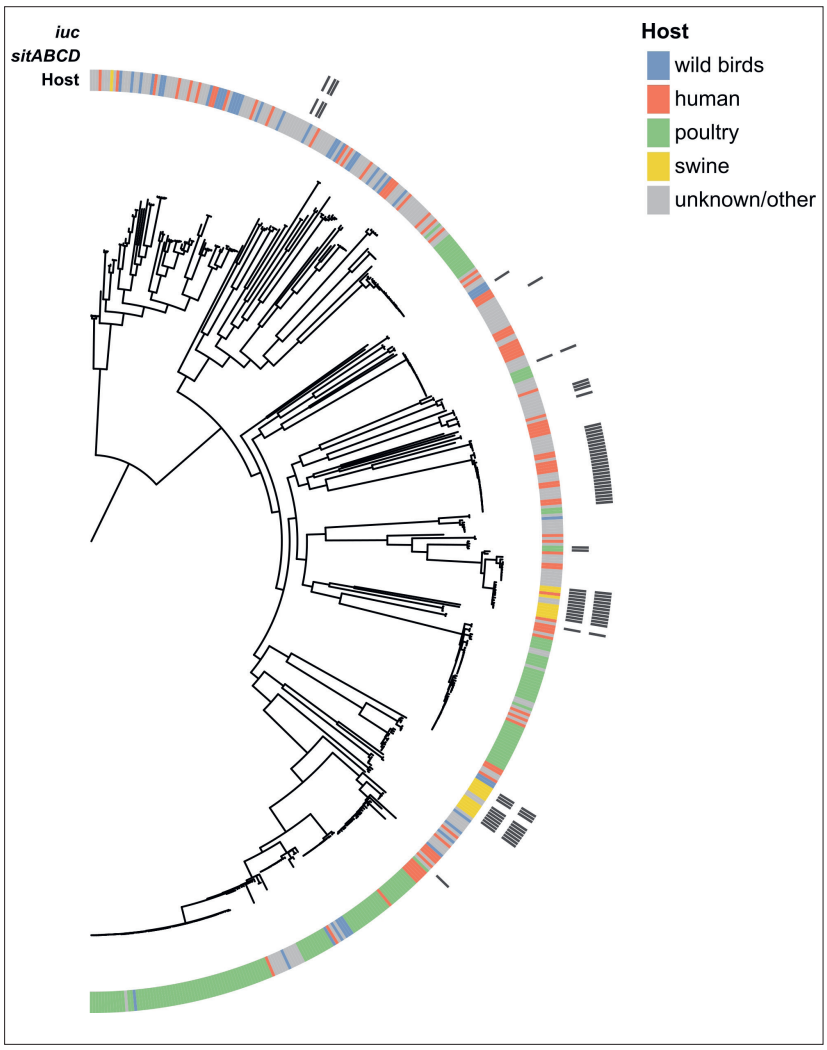

Figure 1: Maximum-likelihood phylogenetic tree of 499 *E. albertii* isolates. The tree is based on 47417 informative sites in a 1.04 Mb core genome alignment. Each isolate is annotated with its origin and the presence of the *iuc* gene cluster and *sitABCD* operon. The tree was visualized using iTOL.[25]

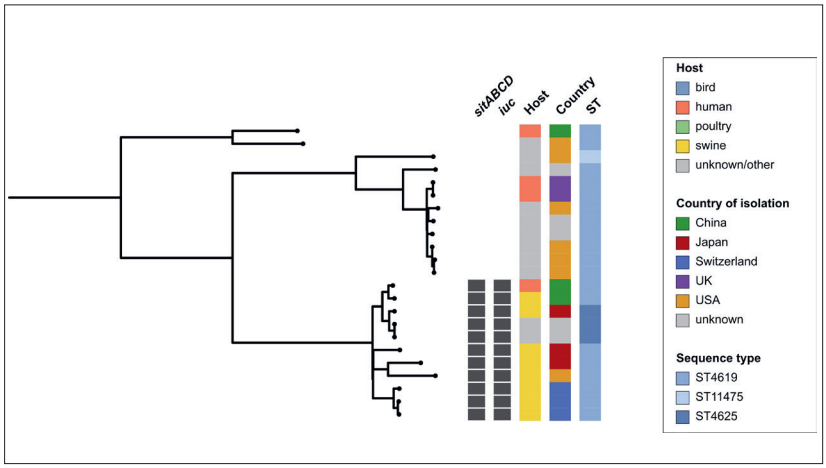

Figure 2: Maximum-likelihood phylogenetic tree of 23 ST4619 group isolates. The tree is based on 3210 informative sites in a 2.44 Mb core genome alignment. Each isolate is annotated with its origin, the presence of the *iuc* gene cluster and *sitABCD* operon, and the sequence type (ST). The tree was visualized using iTOL.[25]

isolates carried two intact aminoglycoside resistance genes: *aph(3")-Ib* (located adjacent of a truncated *aph(6)-Id*) and *aadA1*. Of the five ST2087 isolates, one (KBSW184i) carried resistance genes (*aadA1* and *satA2* [encoding streptothricin resistance]). None of the isolates harboured *stx*.

We subsequently compared the 8 genomes to 491 publicly available *E. albertii* whole genome sequences. These included 11 swine isolates obtained between 1983 and 2018 in China, Japan, and the US. Ten of the 11 global swine isolates clustered with the Swiss swine isolates either in the ST2087 group (n = 5) or in a specific subclade of the ST4619 group (n = 5) (Figure 1), which also encompassed ST4625 and ST11475 isolates (Figure 2). Overall, 12 out of 14 (85,7 %) ST2087 isolates and 8 out of the 11 (72,7 %) ST4619 subclade isolates were associated with swine. This host-associated clustering contrasts with the phylogenetic distribution of *E. albertii* isolates from humans and wild birds, which belonged to diverse *E. albertii* lineages (Figure 1).

Out of 524 virulence genes detected in at least one of the 499 genomes, the *sitABCD* operon (encoding *Salmonella* iron transporter) and the *iuc* gene cluster (encoding aerobactin biosynthesis) were most significantly associated with swine isolates (Figure 1). Long-read sequencing of two isolates (KBSW16i [ST4619] and KBSW50i [ST2087]) demonstrated the co-location of the *iuc* and *sit* genes on structurally similar plasmids (148 kb [F4:A-:B1] and 126 kb [F115:A-:B1], respectively) (Figure 3). The plasmids also contained a *sepA*-like gene (92,5 % amino acid identity to SepA [*Shigella* extracellular protein A]), which was however not present in all *iuc*- and *sit*-positive isolates.

Discussion

Racoons, poultry, and wild birds are recognized as major reservoirs for *E. albertii*.[6,7,13,21,28,45] Here, we demonstrate that swine constitute an important *E. albertii* reservoir in Switzerland. Overall, 23,7 % of all individuals and more than half of all investigated herds yielded positive PCR results. A previous study in Japan detected *E. albertii* in 1/45 healthy swine, suggesting regional differences in positivity rates.[15] In agreement with previous livestock screenings,[20,36,44] we did not detect *E. albertii* in sheep samples. *E. albertii* prevalence in cattle and calves was very low, with only one positive from 200 samples.

In our study, the recovery rate of isolates from PCR-positive pig samples was 16 % (8/51). The low recovery rate is comparable to several studies that have reported challenges in isolating *E. albertii*.[18,19,30] Other species such as *Providencia stuartii* are morphologically indistinguishable from *E. albertii* (colourless to grey-white colonies) and there is very often a very high background flora of *E. coli* (red colonies) on the utilized selective medium complicating the recovery

of *E. albertii*. Improved selective media are therefore needed for the future to enable comprehensive epidemiological and clinical investigations.

Unexpectedly, all Swiss swine isolates and most swine isolates collected over the past 40 years in China, Japan, or the US grouped in one of two phylogenetic clusters (ST2087 and a ST4619 group subclade). The Swiss isolates were obtained from different herds and separated by ≥ 15 SNPs, suggesting no recent inter-herd transmission. The two swine-associated clusters were distantly related but characterized by the shared presence of a virulence plasmid harboring the *sitABCD* and *iuc* genes. The *iuc* and *sitABCD* genes play an important role in iron acquisition.[27] Whether the observed host preference is mediated by virulence factors or by other, possibly independent adaptions remains to be investigated. Contaminated swine feed or transmission from wild animals as source of strains can also not be excluded. The swine-associated clusters encompassed only one human isolate, suggesting a limited zoonotic infection risk. A similar host-associated divergent clustering of *E. albertii* isolates has recently been reported for *E. albertii* isolates from humans and wild birds in the UK.[7]

Conclusion

In conclusion, our study suggests that fattening swine are a reservoir for specific *E. albertii* lineages. Although, foodborne outbreaks in humans have so far only been described in Japan and were not linked to meat,[3,4,24,29,32,36,37] systematic surveillance of *E. albertii* disease has been hindered by misidentification. Pork must therefore be considered as a potential vector for the transmission of *E. albertii* to the consumers.

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Conflict of Interest
None declared.

Author contributions
Karen Barmetter; Formal analysis (lead); Investigation (lead); Methodology (lead); Visualization (supporting); Data curation (lead); Writing-original draft (equal); Writing-review & editing (supporting). Michael Biggel: Formal analysis (supporting); Methodology (lead); Visualization (equal); Writing-original draft (equal); Writing-review & editing (supporting). Francis Muchaamba: Methodology (supporting); Writing-review & editing (supporting). Andrea Treier: Methodology (supporting); Writing-review & editing (supporting); Roger Stephan: Conceptualization (lead); Formal analysis (equal); Investigation (equal); Project administration (lead); Supervision (lead); Writing-original draft (equal); Writing-review & editing (equal).

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Data Availability Statement
Sequencing data generated are available at NCBI under BioProject PRJNA879956.

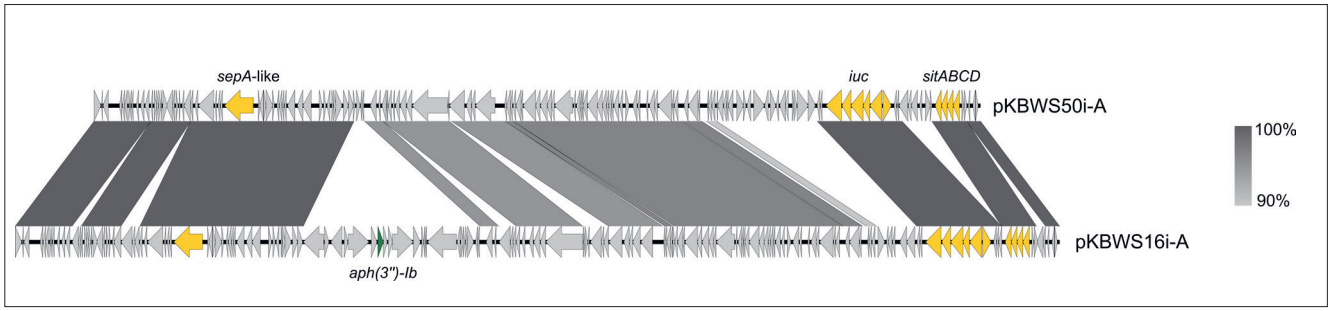

Figure 3: Comparison of the *E. albertii* virulence plasmids pKBSW50i-A and pKBSW16i-A. Virulence genes (yellow) and resistance genes (red) are highlighted. Shaded boxes indicate homologous regions. Sequence comparisons were performed using EasyFig.[42]

Livestock as possible reservoir of *Escherichia albertii* in Switzerland

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Le bétail comme réservoir possible d'*Escherichia albertii* en Suisse

Escherichia albertii est un pathogène alimentaire zoonotique émergent. Sa prévalence, sa distribution et ses réservoirs ne sont pas encore clairement définis. Dans cette étude, nous avons évalué l'occurrence et les caractéristiques génomiques d'*E. albertii* chez le bétail en Suisse. Au total, 515 échantillons cæcaux d'ovins, de bovins, de veaux et de porcs d'engraissement ont été prélevés entre mai 2022 et août 2022 au niveau de l'abattoir. En utilisant une PCR spécifique à *E. albertii* ciblant le gène *Eacdt*, 23,7 % (51/215) des porcs provenant de 24 exploitations différentes étaient positifs. Un (1 %) veau sur 100 a présenté un résultat positif à la PCR, tandis que tous les échantillons d'ovins et de bovins étaient négatifs à la PCR. Huit isolats d'*E. albertii* ont pu être récupérés à partir d'échantillons provenant de porcs et ont été analysés par séquençage du génome entier. Les huit isolats appartenaient au groupe ST2087 ou à un sous-clade du groupe ST4619, comme la plupart des génomes des 11 isolats porcins mondiaux disponibles dans les bases de données publiques. Ces deux groupes partageaient la présence d'un plasmide de virulence hébergeant les gènes *sitABCD* et *iuc*. En résumé, nous démontrons que les porcs d'engraissement constituent un réservoir d'*E. albertii* en Suisse et décrivons des lignées spécifiques associées aux porcs.

Mots clés: *Escherichia albertii*, porc, porcs, veaux, bovins, moutons, réservoir

L'allevamento di animali da reddito come possibile serbatoio di *Escherichia albertii* in Svizzera

La *Escherichia albert*ii è un patogeno zoonotico emergente negli alimenti. La sua prevalenza, distribuzione e i serbatoi non sono stati ancora chiaramente identificati. In questo studio abbiamo valutato la presenza e le caratteristiche genomiche di *E. albertii* negli animali da allevamento in Svizzera. Tra il maggio e l'agosto 2022, un totale di 515 campioni cecali sono stati raccolti da ovini, bovini, vitelli e suini da ingrasso durante il processo di macellazione. Utilizzando una PCR specifica per *E. albertii* mirata al gene *Eacdt*, il 23,7 % (51/215) dei suini provenienti da 24 aziende diverse è risultato positivo. Un vitello (1 %) su 100 ha mostrato un risultato positivo alla PCR, mentre tutti i campioni di ovini e bovini sono risultati negativi alla PCR. È stato possibile ottenere otto isolati di *E. albertii* da campioni di suini e sono stati analizzati mediante il sequenziamento dell'intero genoma. Tutti gli otto isolati appartenevano al gruppo ST2087 o a un sottoclade del gruppo ST4619, così come la maggior parte dei genomi degli 11 isolati globali di suini disponibili nelle banche dati pubbliche. Questi due cluster condividono la presenza di un plasmide di virulenza che ospita i geni *sitABCD* e *iuc*. In conclusione, abbiamo dimostrato che i suini da ingrasso rappresentano un serbatoio di *E. albertii* in Svizzera e abbiamo descritto specifici lignaggi associati ai suini

Parole chiave: *Escherichia albertii*, maiale, suino, vitelli, bovini, ovini, serbatoio

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