RESEARCH ARTICLE

Carbapenem-resistance and pathogenicity of bovine Acinetobacter indicus-like isolates

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Abstract

The objective of this study was to characterize blaOXA-23 harbouring Acinetobacter indicus-like strains from cattle including genomic and phylogenetic analyses, antimicrobial susceptibility testing and evaluation of pathogenicity in vitro and in vivo. Nasal and rectal swabs (n = 45) from cattle in Germany were screened for carbapenem-non-susceptible Acinetobacter spp. Thereby, two carbapenem resistant Acinetobacter spp. from the nasal cavities of two calves could be isolated. MALDI-TOF mass spectrometry and 16S rDNA sequencing identified these isolates as A. indicus-like. A phylogenetic tree based on partial rpoB sequences indicated closest relation of the two bovine isolates to the A. indicus type strain A648T and human clinical A. indicus isolates, while whole genome comparison revealed considerable intraspecies diversity. High minimum inhibitory concentrations were observed for carbapenems and other antibiotics including fluoroquinolones and gentamicin. Whole genome sequencing and PCR mapping revealed that both isolates harboured blaOXA-23 localized on the chromosome and surrounded by interrupted Tn2008 transposon structures. Since the pathogenic potential of A. indicus is unknown, pathogenicity was assessed employing the Galleria (G.) mellonella infection model and an in vitro cytotoxicity assay using A549 human lung epithelial cells. Pathogenicity in vivo (G. mellonella killing assay) and in vitro (cytotoxicity assay) of the two A. indicus-like isolates was lower compared to A. baumannii ATCC 17978 and similar to A. lwoffii ATCC 15309. The reduced pathogenicity of A. indicus compared to A. baumannii correlated with the absence of important virulence genes encoding like phospholipase C1+C2, acinetobactin outer membrane protein BauA, RND-type efflux system proteins AdeRS and AdeAB or the trimeric autotransporter adhesin Ata. The emergence of carbapenem-resistant A. indicus-like strains from cattle carrying blaOXA-23 on transposable elements and revealing genetic relatedness to isolates from human clinical sources requires further investigations regarding the pathogenic potential, genomic characteristics, zoonotic risk and putative additional sources of this new Acinetobacter species.
Introduction

*Acinetobacter baumannii* is an opportunistic pathogen frequently involved in a wide range of nosocomial infections [1]. For more than a decade now, carbapenem-resistant *Acinetobacter* spp. strains, particularly *A. baumannii*, represent a growing public health concern, since they often confer resistance to other critically important antimicrobials, including aminoglycosides, fluoroquinolones or even polymyxins [2, 3]. Carbapenem resistance in *Acinetobacter* spp. is most often mediated by oxacillinases (OXA) which belong to the group of carbapenem-hydrolyzing class D β-lactamases (CHDLs). The most prevalent OXA carbapenemases found in *Aci- netobacter* are acquired OXA-23 and OXA-58 which can be either plasmid or chromosome encoded. OXA carbapenemases exhibit only weak hydrolysis of carbapenems *in vitro* but are often associated with insertion sequences that provide additional promoter elements leading to overexpression of CHDLs and finally to carbapenem resistance in clinical isolates [1–3]. Although reports about carbapenem resistant *Acinetobacter* spp. strains in animals are still infrequent, they have been increasing in the last few years. OXA-23 was identified in *A. variabilis* from cattle in France [4], in *A. gandensis* strains from horses in the Netherlands [5], and in *A. baumannii* from cats in Portugal and Germany [6–8]. Different carbapenemases were further recovered in *A. baumannii* from livestock animals in Lebanon [9], and from swine in China [10]. In 2012, a novel species, termed *A. indicus*, has been identified from a cyclohexane-containing dumpsite [11]. Two years later Bonnin et al. reported OXA-23 mediated carbapenem resistance in a human clinical isolate identified to be closely related to this species and, thus, was termed *A. indicus*-like [12].

In the present study, two carbapenem-resistant, OXA-23 carrying *Acinetobacter indicus*-like isolates were recovered from nasal swabs of two calves. We characterized the genetic environment of *bla*OXA-23, and performed genomic and phylogenetic analyses in order to get insights into acquisition and dissemination. Since the clinical relevance of *A. indicus* is unknown, we evaluated pathogenicity *in vitro* and *in vivo* in comparison to reference strains of *A. baumannii* and the closely related *A. lwoffii*.

Materials and methods

Bacterial strains, species identification and assignment to international clones

From September 2014 to March 2015 nasal and rectal swabs as well as composite fecal samples from the corresponding stables (n = 45) were taken from cattle (*Bos taurus*) in Hesse (coordinates 50°39’58”N 8°35’28”E), Germany. Cattle breeds included Holstein-Frisian, Angus, Hereford, Swiss-Brown, Pinzgauer and Vogelsberger Rotes Höhenvieh. The samples were cultured on blood agar (blood agar base by Merck Chemicals, Darmstadt, supplemented with 5% sheep blood) and on Gassner agar (Oxoid, Wesel, Germany). Screening for carbapenem-non-susceptible *Acinetobacter* spp. was done by using Mueller-Hinton agar plates (Oxoid, Wesel, Germany) containing 2 mg/L and 4 mg/L meropenem (Sigma-Aldrich, Munich, Germany), respectively. Colonies with suspected reduced susceptibility to carbapenem were initially identified at the species level using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Bruker Daltonics, Bremen, Germany). Species identification was verified by multiplex PCR targeting different portions of the *gyrB* gene and by 16S rRNA gene sequence analysis [13].
Whole genome sequencing and phylogenetic analysis and screening for virulence-related genes

For whole genome sequencing of two bovine A. indicus strains, DNA was extracted with the “Master Pure™ DNA Purification Kit” (Biozym Scientific GmbH, Hessisch Oldendorf, Germany). Genome sequencing was done using an Illumina MiSeq sequencer with multiplexing of 30 samples per flow cell using 300 bp paired-end reads and a minimum of 50-fold coverage. Sequence data were assembled de novo using SPAdes Genome Assembler V. 3.8 [14]. Phylogenetic analysis was initially performed by using the partial rpoB sequence of A. indicus-like strains and comparing them with publicly available rpoB sequences of type or reference strains of known species of the genus Acinetobacter. Similarity calculations and cluster analysis were carried out for a 823 bp region spanning nucleotide positions 2944–3766 of the rpoB coding region of A. baumannii CIP70.34 T. MAFFT (Multiple Alignment using Fast Fourier Transform) alignment was performed to cluster partial rpoB sequences [15]. A PhyML (Phylogenetic software based on the Maximum-Likelihood principle) tree was created using the HKY85 substitution model and bootstrap values were determined after 1000 simulations using the Generous 8.1.3 software (Biomatter Ltd., Auckland, New Zealand) [16].

Phylogenetic relationships were further determined on the basis of the Maximum Common Genome (MCG) [17], which represents the set of orthologous genes that are present in all genomes under study. In a first comparison, we included 32 publicly available representatives of the different Acinetobacter spp. (S1 Fig). Secondly, we compared the genomes of six A. indicus strains (including three genome sequences of strain A648T, which were submitted under different labels), namely IHIT27599 (accession number MRUS00000000), IHIT27630 (MRUT00000000), KM7 (JZRF01000070), CIP 110367 (= A648T; ACET00000000.1), ANC 4215 (= A648T; ATGH00000000.1), and DSM 25388 (= A648T; BBSF00000000.1). A prediction of genes was performed by using the Prokaryotic Dynamic Programming Genefinding Algorithm For Microbial Genomes (Prodigal) [18]. The coding sequences where subsequently clustered using USEARCH v7 [19] based on a threshold of 70% similarity on nucleotide level and 90% coverage to determine the set of orthologous genes of all genomes included in the respective comparison. Using these sets as a reference, we extracted the corresponding allelic variants of the MCG genes from the genomes (32 and 6, respectively) using PLAST v2.3.1 [20], aligned them with MUSCLE v3.8.31 [21] and finally concatenated them. The resulting alignment was used to infer a maximum likelihood phylogeny using RAxML version 8.1.14 with a General Time Reversible model and gamma correction for among site rate variation [22].

Antimicrobial susceptibility and resistance genes

Antimicrobial susceptibility was determined by antibiotic gradient tests (Liofilchem, Roseto degli Abruzzi, Italy). Minimum inhibitory concentrations (MICs) were interpreted according to breakpoints defined for human Acinetobacter spp. by either EUCAST or CLSI [23, 24]. MICs of tigecycline and chloramphenicol were interpreted according to breakpoints for Enterobacteriaceae set by EUCAST [23]. Whole genome sequences were used to identify resistance genes by using the online tool ResFinder 2.1, provided by the Center for Genomic Epidemiology (CGE) (http://www.genomicepidemiology.org/). PCR mapping of the genetic environment of blaOXA-23 was performed to determine the correct order of contigs and sequence assemblies using primers listed in S1 Table. The genetic location of the blaOXA-23 gene was evaluated by performing I-CeuI digestion of whole-cell DNAs followed by Southern blot hybridization using 16S rRNA and blaOXA-23 probes as previously reported [25, 26].
Analysis of pathogenicity in the *Galleria mellonella* infection model

Pathogenicity of *Acinetobacter* spp. strains was analysed employing last-instar larvae of the greater wax moth (*Galleria mellonella*) [27]. A 1:50 dilution of an overnight bacterial culture in lysogeny broth (LB) was prepared and grown to an OD$_{600}$ of 1.0 at 37˚C. A phosphate-buffered saline (PBS) solution containing serial dilutions of this culture representing colony forming units of $5 \times 10^2$ to $5 \times 10^6$ was injected into the last left proleg of the larvae using a Hamilton precision syringe. PBS solution alone served as negative control. Upon infection, larvae were incubated in petri dishes at 37˚C for 72 h and scored for survival by two independent observers daily. For determination of the median lethal dose (LD$_{50}$), a series of 10-fold serial dilutions were injected and LD$_{50}$ were calculated after 24 h by nonlinear regression analysis using GraphPad Prism 5.0 (La Jolla, USA) as described [28, 29].

Cell viability assay

A549 human lung epithelial cells (ATCC® CCL-185) were grown in six well plates in Dulbecco’s Modified Eagle Medium (DMEM; Biochrom GmbH, Berlin, Germany) with 10% foetal calf serum (FCS; Biochrom GmbH, Berlin, Germany) at 37˚C until almost confluent. Different *Acinetobacter* spp. were used at a multiplicity of infection (MOI) of 100 and incubated for 20 h. Thereafter, the supernatant was filtered (0.45 µm) and lactate dehydrogenase (LDH) activities were determined by spectrophotometry at a wavelength of 340 nm using the IFCC method as described by Schuman et al. [30]. The detergent Triton X-100 (0.1% in PBS) and DMEM were used as positive and negative controls, respectively. Mean LDH values of medium-treated A549 cells versus infected cells were analysed by an unpaired two-tailed Student’s t test (GraphPad Prism 5.0). A p value of $\leq 0.05$ was considered statistically significant, and a p value of $\leq 0.001$ was considered highly significant.

Screening for virulence-related genes

Screening for virulence-related genes was performed by using the online tool MyDbFinder 1.1, provided by the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/). Only genes that have previously been associated with one of the phenotypes investigated later, i.e. killing of *G. mellonella* larvae and cytotoxicity to human lung epithelial cells, were included. Using the Geneious 8.1.3 software a MAFFT alignment was performed to cluster nucleotide/amino acid sequences and to calculate sequence identity to a given reference sequence.

Results

Susceptibility of bovine *Acinetobacter* spp. isolates to carbapenems

During screening of cattle for carbapenem-non-susceptible *Acinetobacter* spp. two isolates showed growth on the meropenem-containing screening agar. Strain IHIT27630 was isolated in September 2014 from the nasal cavity of a calf which was hospitalized in a veterinary clinic due to dermatophytosis, wasting syndrome, diarrhoea and bronchopneumonia. The second strain, IHIT27599, was isolated one month later from the nasal cavity of a calf which was suffering from bronchopneumonia and omphalophlebitis. This calf was sampled on a farm 35 miles away from the veterinary clinic and nearly 60 miles apart from the farm where the first calf originated from, making an epidemiologic link and a transmission highly unlikely. Using MALDI-TOF MS analysis, both isolates were presumptively suggested as *A. calcoaceticus* but with low reliability (score values of 1.57 and 1.56 using the IVD MALDI Biotyper library). 16S rDNA sequence analysis enabled a more precise identification and indicated a high homology (>99%) to published sequences of *A. indicus* and *A. indicus*-like strains, including strain RAB1.
and the type strain A648\textsuperscript{T}. RAB1 originates from a human rectal swab and was isolated in France in 2011, whereas A648\textsuperscript{T} was obtained from a cyclohexane dumping site in India sometime before 2010 [12, 31].

**Phylogenetic analysis and genome comparison**

A phylogenetic tree was compiled based on the alignment of partial 823-bp \textit{rpoB} sequences from the two bovine \textit{A. indicus}-like isolates, 34 distinct \textit{Acinetobacter} spp. with validly published names, seven \textit{Acinetobacter} spp. with effectively published names awaiting validation (www.bacterio.net), and six \textit{A. genomospecies} strains. The analysis indicated closest relation between IHIT27630 and IHIT27599 and the \textit{A. indicus} type strain A648\textsuperscript{T} with 97.69% nucleotide sequence similarity of partial \textit{rpoB} sequence (Fig 1). The next closest related strains were \textit{A. guangdongensis} strain 1 NM-4\textsuperscript{T} (92.22%), \textit{A. variabilis} ANC 4750 (87.97%), \textit{A. genomospec} 15TU (now \textit{A. variabilis}) NIPH 546 (86.76%), and \textit{A. lwoffii} CIP 61.10\textsuperscript{T} (86.39%), while \textit{A. qingfengsis} strain 2BJ1\textsuperscript{T} revealed the least closely related \textit{rpoB} sequence (76.01%). When generating a phylogenetic tree employing all published \textit{rpoB} sequences from \textit{A. indicus}-like strains (accessed at 29th December 2016), our bovine isolates clustered with human clinical \textit{A. indicus}-like strain LUH10523 (99.88% nucleotide sequence identity) that was obtained from the blood culture of a patient in The Netherlands in 2005 (Fig 2) [12]. Next closely related was \textit{A. indicus}-like strain CIP 53.82 that was obtained from a human patient with postoperative meningitis in 1953 in France [32, 33]. Overall, the \textit{rpoB} regions showed intraspecies similarity values for the 11 strains, ranging between 96.36% and 100%. There was no evidence for a separation of environmental (strains KM7 and A648\textsuperscript{T}), animal (isolates from the present study and strains LUH08556 + LUH8511 from cow faeces) and human strains (LUH05836, RAB1, LUH05041 and LUH10523) or of strains with or without carbapenemases [12]. Apart from our study isolates, only \textit{A. indicus}-like strain RAB1 expressed the OXA-23 carbapenemase [12]. In addition, in silico analysis of the genome sequence of \textit{A. indicus}-like strain CIP 53.82 (acc. no. APRK00000000.1) revealed the presence of the \textit{blaOXA-58} gene, which is flanked by two incomplete \textit{ISAb} insertion elements [32, 33].

For a higher phylogenetic resolution, we further compared 32 isolates of different \textit{Acinetobacter} spp. based on genome sequences (S1A Fig). As whole genomes of several species included in the \textit{rpoB}-based tree (Fig 1) were not available, a direct comparison of both tree phylogenies was not possible. The MCG of the 32 selected \textit{Acinetobacter} spp. isolates revealed 42 orthologous genes and the alignment of the genes consisted of 26,161 sites from which 8,026 were informative SNP sites. Pairwise distance varied between 0 and 4,049 sites. Similar to what has been observed for the \textit{rpoB}-based tree, the \textit{A. indicus} strains clustered together and strains of the species \textit{A. towneri}, \textit{A. tandoii} were placed in closer vicinity as for example those of \textit{A. baumannii} and \textit{A. pittii}, as shown in S1A Fig and verified by pairwise distance values (S2 Table).

A separate calculation of the MCG for the \textit{A. indicus} strains revealed 2,145 orthologous genes and the alignment of these genes consisted of 2,027,793 sites from which 109,137 were phylogenetically informative SNP sites. Based on this analysis, the genome sequences submitted under three different labels and accession numbers for \textit{A. indicus} type strain A648\textsuperscript{T} differed by 313 (ANC 4215 versus CIP 110367) to 1917 (CIP 110367 versus DSM 25388) SNPs, which may be due to different sequencing strategies or strain material. Apart from this, hardly any similarity-based clustering could be observed, suggesting a considerable diversity between the genomes of strains A648\textsuperscript{T}, KM7, IHIT27599 and IHIT27630. This was also evident in the pairwise distance which varied between 64,841 and 86,573 SNPs to the type strain (S2B Table). Even the two bovine IHIT strains differed by 35,396 from each other, clearly indicating two different strains.
Fig 1. Neighbour-joining phylogenetic tree based on partial nucleotide sequences of the rpoB (823 bp) genes of Acinetobacter indicus-like strains IHIT27630 and IHIT27599 and 47 type or reference strains of known Acinetobacter species. The tree was constructed using the maximum likelihood method. Bootstrap values (>50%) after 1,000 simulations are shown at branch nodes. GenBank accession nos. are given in parentheses. Bar, 0.2 nucleotide substitutions per site.

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Antimicrobial susceptibility and resistance genes

The two bovine *A. indicus*-like isolates showed high MICs to imipenem, meropenem and doripenem (Table 1) and represent the first known carbapenem-resistant *A. indicus*-like isolates of animal origin. While the isolates remained susceptible to third and fourth generation cephalosporins, non-susceptibility was observed for a number of other antimicrobial substances, including piperacillin-tazobactam, fluoroquinolones, gentamicin, and doxycycline (Table 1).

The two isolates differed slightly in their antimicrobial resistance profile with IHIT27599 being additionally resistant to tobramycin and co-trimoxazole. Sanger sequencing confirmed that both isolates harboured the \(\beta\)-lactamase OXA-23 which is widespread in *A. baumannii* [34]. Subsequent whole genome sequencing revealed the presence of aminoglycoside resistance genes \(aac(3)-\text{IIa}\), \(strA/B\) and \(aph(3')-\text{Ic}\), sulfonamide resistance gene \(sul2\), phenicol resistance gene \(floR\), and tetracycline resistance genes \(tet(A)\) and \(tet(Y)\) in isolate IHIT27630. The
The second isolate (IHIT27599) possessed aminoglycoside resistance genes *aadA1*, *aadB*, *strA/B* and *aph(3′)-lc*, sulfonamide resistance genes *sul1* and *sul2*, phenicol resistance gene *floR*, and tetracycline resistance genes *tet(X)* and *tet(Y)*, which is in line with the phenotypic results.

**Localization and genetic environment of *bla*<sub>OXA-23</sub>**

I-CeuI digestion of whole-cell DNAs and Southern blot hybridization using 16S rRNA and *bla*<sub>OXA-23</sub> probes demonstrated that both *A. indicus*-like isolates harboured *bla*<sub>OXA-23</sub> on the chromosome. Genome sequencing and PCR mapping of assembled contigs identified a differently interrupted and incomplete *Tn2008* transposon structure surrounding the *OXA* genes (Fig 3). In both isolates the same genetic structure is present downstream of *bla*<sub>OXA-23</sub>, including a putative AAA <sub>ATPase</sub> gene disrupted by a partial insertion sequence *ISAcra1* of 564 bp in length and the transcriptional regulator gene *merR*. We identified a 105-bp long *ISAba1*-remnant which was either truncated by a full version of insertion sequence *ISAcsp2* in isolate...
IHIT27630 or by a partial ISAcsp2 sequence which was preceded by IS26 in isolate IHIT27599. In case of IHIT27630, a second disrupted copy of ISAcsp2 was preceded by a full-length copy of a Tn2008-related ISAbal transposase gene and the remaining 168-bp sequence of the aforementioned truncated ISAcra1. ISAcra1 is a novel insertion element that has previously been described in an A. radioresistens strain where it was flanked by a typical 7-bp direct repeat (DR) / insertion site (ATTATAT) as well as a 15-bp inverted repeat left (IRL; GGCTCTAGAC TAGCA) and inverted repeat right (IRR; TGCTAGTCTAGAGCC) [35]. In isolate IHIT27630, the disrupted ISAcra1 element lacked the downstream DR, whereas the characteristic upstream DR and the two IR regions were identical to those described recently [35]. In both isolates, 27 nucleotides are present between insertion sequence ISAbal and the start codon of blaOXA-23, which is typical of Tn2008 in contrast to the previously described Tn2008b [36].

Different genetic structures associated with the blaOXA-23 genes in the two A. indicus isolates supports previous reports about the high variability of this flanking region in A. baumannii. In addition, the finding of ISAcra1, although disrupted, may be a hint towards A. radioresistens as original source of the blaOXA-23 gene not only in A. baumannii but also in A. indicus [37].

Virulence properties of A. indicus-like strains

Clinically relevant biological features of A. indicus remain totally elusive. In order to evaluate the virulence of IHIT27599 and IHIT27630, the Galleria mellonella in vivo infection model was employed. G. mellonella larvae were infected with different infection doses of the two bovine A. indicus-like isolates and reference strains of A. baumannii (ATCC 17978) and A. lwofii (ATCC 15309), which were included for comparative analysis. Infection of larvae with the different Acinetobacter spp. caused a time- and dose-dependent killing of larvae (Fig 4). Whereas almost no mortality was observed when 5x10^4 cfu of A. indicus-like species were injected, almost 100% of G. mellonella larvae died 72 h post infection with 5x10^6 cfu (Fig 4C and 4D). Injection of the highest dose of 5x10^6 A. lwofii ATCC 15309, which was selected due to its close phylogenetic relatedness to A. indicus (Fig 1), resulted in death of approximately 40% of
larvae after 72 h. In contrast, injection of only 5x10^4 A. baumannii ATCC 17978 resulted in the death of 50% of larvae 24 h post infection. We determined median lethal doses (LD50) to compare virulence across the Acinetobacter strains (S3 Table). A. indicus-like strains IHIT27630 and IHIT27599 displayed LD50 values of 5.67 [95% CI 5.51–5.82] and 6.20 [95% CI 5.91–6.50] and, thus, were more virulent than A. Iwoffii ATCC 15309 with an LD50 of 6.84 [95% CI 6.65–7.04]. A. baumannii ATCC 17978 displayed the lowest LD50 of 4.72 [95% CI 4.42–5.01] and was therefore the most virulent of all tested strains in the Galleria in vivo infection model.

To investigate the capability to disrupt membrane integrity of human cells, A549 lung epithelial cells were infected with the same Acinetobacter strains used in the Galleria assay (Fig 5). Infection with A. baumannii ATCC 17978 resulted in a LDH release of 100 U/L. In contrast, infection with A. Iwoffii ATCC 15309 or the two A. indicus-like strains induced a LDH release of approximately only 25 U/L (range 22.2–27.9 U/L) compared to the medium control (DMEM) (16.3 U/L) (p = 0.0026 for A. Iwoffii; p = 0.0709 for IHIT27599; p = 0.20 for IHIT27630). This indicates that cytotoxicity towards A549 human lung epithelial cells of Fig 4. Dose-dependent lethality of Galleria mellonella infected with Acinetobacter spp. strains. Larvae were injected with different cfu (5x10^2 to 5x10^6) and survival was monitored over 72 h after infection. Mean values from at least four experiments are shown. Error bars show standard error of the mean.

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the three Acinetobacter non-baumannii strains was comparable to each other but much lower compared to A. baumannii ATCC 17978 (3.6 to 4.5-fold lower LDH activities; \( p < 0.001 \)).

**Presence of virulence genes related to** in vivo **virulence and** in vitro **cytotoxicity**

We screened the genomes of the strains A. baumannii ATCC 17978, A. lwoffii ATCC 15309, A. indicus IHIT27599 and A. indicus IHIT27630, all of which were included in phenotypical assays, for virulence genes that have previously been linked with killing of G. mellonella larvae and cytotoxicity to human epithelial cells. A. baumannii ATCC 17978 harboured most of the virulence determinants linked with the above mentioned phenotypes and only lacked the phospholipase D1 gene pld1 and DNA uptake protein genes comEC (Table 2). In contrast, A. lwoffii strain ATCC 15309 and the two A. indicus strains IHIT27599 and IHIT27630 harboured only some of the tested virulence-associated determinants but lacked for example phospholipase genes plc1 and plc2, acinetobactin outer membrane receptor protein gene bauA, efflux pump system protein genes adeRS and adeAB, and the trimeric autotransporter adhesion gene ata. In case orthologous proteins could be identified in the A. indicus strains, they revealed amino acid sequence identity to the A. baumannii reference genomes ranging from 47.6% for SurA1, 54.4% for PLD1, and 62.6% for Omp33 to a maximum of 91.4% for SodB (Table 2).
Since its first description as a novel environmental *Acinetobacter* species in 2012, very few studies reported about the isolation of *A. indicus*-like strains from different sources [12, 33, 38]. Difficulties in discriminating this novel species, together with its unknown clinical relevance might be reasons for that. Based on MALDI-TOF MS analysis, which has been shown to be a useful tool for identification of *Acinetobacter* spp. [32], our bovine isolates were initially misclassified as *A. calcoaceticus*, albeit with unreliable score values. Having similar problems with the species designation based on biochemical properties, Bonnin *et al.* (2014) established MALDI-TOF MS reference spectra for reliable identification of *A. indicus*-like isolates. Using these new spectra, two animal and three human *A. indicus*-like isolates from the Netherlands and Belgium, which were identified by *rpoB* sequencing and 16S rRNA analysis, grouped together but were clearly distinct from the type strain A648T [12]. This was also evident from a genome-based comparison, where the two bovine *A. indicus*-like strains from the present study clustered together, but were clearly separated from the two environmental *A. indicus* strains A648T and KM7. Future genomic studies, including a broader set of *A. indicus*-like

### Table 2. Presence and absence of virulence gene/protein orthologs in four *Acinetobacter* species strains used for virulence assays.

| Virulence determinant                  | Gene | Gene size in reference sequence (bp) | Gene position in reference sequence* | Nucleotide/amino acid sequence similarity to reference sequence* (%) |
|----------------------------------------|------|--------------------------------------|--------------------------------------|---------------------------------------------------------------|
|                                        |      |                                      |                                      | *A. baumannii ATCC 17978| *A. lwoffii ATCC 15309| *A. indicus IHIT27599| *A. indicus IHIT27630|
| Phospholipase C1                       | plc1 | 2229                                 | 1575962..1578190                    | 100/100 | - | - | - |
| Phospholipase C2                       | plc2 | 2169                                 | 3794856..3792688                    | 100/100 | - | - | - |
| Phospholipase D1                       | pld1 | 1527                                 | 140863..142389                      | - | 57.1/50.3 | 58.6/54.4 | 58.6/54.4 |
| Phospholipase D2                       | pld2 | 1626                                 | 524407..522783                      | 100/100 | 64.2/70.4 | 65.9/67.1 | 65.9/67.1 |
| Phospholipase D3                       | pld3 | 1464                                 | 628527..627064                      | 100/100 | 67.4/69.4 | 67.3/68.4 | 67.2/68.2 |
| Acinetobacitin outer membrane receptor protein | bauA | 2190                                 | 160673..158484                      | 100/100 | - | - | - |
| Type VI secretion system protein       | tssM | 3825                                 | 2445726..2441902                   | 100/100 | 66.0/66.1 | 71.3/77.8 | 71.5/77.7 |
| Autotransporter adhesin                | ata  | 5622                                 | 2775454..2781075                   | 100/100 | - | - | - |
| Surface antigen protein 1              | surA | 318                                  | 2349708..2350025                   | 100/100 | - | 58.2/48.6 | 58.2/48.6 |
| Efflux pump system protein             | adeRS| 1861                                 | 1923981..1931241                   | 100/100 | - | - | - |
| Efflux pump system protein             | adeAB| 4298                                 | 1931387..1935684                   | 100/100 | - | - | - |
| Efflux pump system protein             | arpA | 1101                                 | 40488..41588                        | 100/100 | 66.5/70.6 | 66.9/71.4 | 67.2/71.7 |
| Efflux pump system protein             | arpB | 3126                                 | 41591..44716                       | 100/100 | 73.1/83.2 | 73.4/84.3 | 73.4/84.2 |
| Superoxide dismutase B                 | sod2343 | 627                                 | 1256650..1256024                   | 100/100 | 83.9/89.9 | 84.1/91.4 | 84.4/91.4 |
| DNA uptake channel proteins            | comEC| 4198                                 | 4695..8892                        | - | - | - | - |
| Outer membrane protein A               | ompA | 1071                                 | 680965..682035                     | 100/100 | 81.6/84.3 | 82.6/87.4 | 82.6/87.4 |
| Outer membrane protein 33              | omp33| 900                                  | 212171..211272                     | 100/100 | 66.1/58.8 | 68.8/62.6 | 68.8/62.6 |

*Refers to *A. baumannii* ATCC 17978 (Acc-No. CP012004.1) except for genes *pld1* (*A. baumannii* ATCC 19606; Acc. No. ACQB01000015.1) and *comEC* (*Acinetobacter* species BD413; AF027189.3); minus (-) denotes that the gene/protein is not present in the data source.

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**Discussion**

Since its first description as a novel environmental *Acinetobacter* species in 2012, very few studies reported about the isolation of *A. indicus*-like strains from different sources [12, 33, 38]. Difficulties in discriminating this novel species, together with its unknown clinical relevance might be reasons for that. Based on MALDI-TOF MS analysis, which has been shown to be a useful tool for identification of *Acinetobacter* spp. [32], our bovine isolates were initially misclassified as *A. calcoaceticus*, albeit with unreliable score values. Having similar problems with the species designation based on biochemical properties, Bonnin *et al.* (2014) established MALDI-TOF MS reference spectra for reliable identification of *A. indicus*-like isolates. Using these new spectra, two animal and three human *A. indicus*-like isolates from the Netherlands and Belgium, which were identified by *rpoB* sequencing and 16S rRNA analysis, grouped together but were clearly distinct from the type strain A648T [12]. This was also evident from a genome-based comparison, where the two bovine *A. indicus*-like strains from the present study clustered together, but were clearly separated from the two environmental *A. indicus* strains A648T and KM7. Future genomic studies, including a broader set of *A. indicus*-like
isolates from different sources should help to elucidate the phylogenetic relatedness among members of this novel species more precisely, probably leading to the identification of different genotypic lineages.

Prior to our study, the first and still only published case of carbapenem resistance in an A. indicus-like strain was reported in 2014 by Bonnin et al. [12]. Here, the presence of OXA-23 in a rectal swab isolate (RAB1) from a French patient previously hospitalized in Algeria after a road traffic accident in August 2011 could be demonstrated [12]. In addition, we could identify the carbapenemase gene blaOXA-58 in the genome sequence of the human clinical A. indicus-like isolate CIP 53.82. Despite the presence of blaOXA-58 it is unclear, whether this strain was also phenotypically resistant to carbapenems. When evaluating antimicrobial susceptibilities for human isolate RAB1 and our bovine A. indicus-like isolates, carbapenem MICs ranged from 8 to >32 mg/L, indicating a high level resistance in both cases. Indeed, a strong promoter with distinct -10 and -35 motifs located within the ISAbal sequence was present in our bovine isolates and most likely conferred clinically relevant resistance to carbapenems [39, 40]. Four other previously published A. indicus-like isolates, obtained from cow faeces and from human clinical samples between 1998 and 2003 in the Netherlands did not harbour a carbapenemase gene, nor were they resistant to carbapenems [12].

In contrast to the study of Bonnin et al., where blaOXA-23 of human A. indicus-like strain RAB1 was located on a conjugative plasmid, the bovine isolates from our study harboured the gene on the chromosome [12]. However, as the genomes of A. indicus strains IHIT27599 and IHIT27630 revealed plasmid sequences in the genetic surrounding of blaOXA-23, it may be assumed that the resistance gene was once acquired by horizontal plasmid transfer and subsequent loss of the plasmid, which warrants further investigations. While strain RAB1 possessed ISAbal upstream of the blaOXA-23 gene, which corresponds to transposon Tn2007 [36], the bovine isolates revealed an interrupted Tn2008 with a single ISAbal located upstream of the blaOXA-23 gene, as recently described for other isolates as well [12, 32]. Transposon Tn2008, which is a major vehicle for spreading of blaOXA-23 in A. baumannii [41], was recently also identified in one out of nine OXA-23 positive A. variabilis isolates from cows [4]. In several of these nine isolates, including those with the highest MICs to carbapenems, the ISAbal element of Tn2008 was truncated by a novel insertion sequence termed ISAcsp2 [4]. In our bovine strain IHIT27630 we identified a full copy of ISAcsp2, preceded by an open reading frame encoding a protein of unknown function and a second truncated ISAcsp2. In case of IHIT27599, the truncated ISAbal was directly preceded by a truncated ISAcsp2, indicating, that the genetic context of the blaOXA-23 gene is not unique and probably undergoes evolutionary changes.

We further detected an interrupted version of the recently described 732-bp insertion sequence element ISAcra1 of A. radioresistens in strain IHIT27630 (Fig 3). ISAcra1 has been associated with overexpression of the intrinsic blaOXA-23 in A. baumannii and A. radioresistens strains resulting in phenotypic carbapenem resistance [35]. This novel insertion sequence element has also been associated with the spread of blaOXA-23 in strains of the species A. radioresistens, which is the likely source of this gene [37], and this might be the case for A. indicus isolates as well.

Due to its novelty, barely anything is known about the pathogenicity of members of the species A. indicus. In order to assess pathogenicity of A. indicus in vivo, Galleria mellonella larvae were employed. G. mellonella has recently been described as a non-vertebrate infection model for studying human pathogens, including A. baumannii with regard to pathogenetic and therapeu tic aspects [27]. In our study, both A. indicus-like strains were slightly more virulent compared to A. lwoffii ATCC 15309 since lower LD50 values were obtained and more larvae died when injecting same colony forming units. In contrast, A. baumannii ATCC 17978 was clearly more virulent than all other species in concordance with the LDH assay.
We further employed the LDH assay to monitor cytotoxicity towards human lung epithelial cells in vitro. Here, the two bovine A. indicus-like isolates and A. lwofii displayed an abrogated phenotype in the cell toxicity assays compared to ATCC 17978 since LDH values were only slightly higher than the negative control DMEM. As anticipated, cytotoxicity of A. baumannii ATCC 17978 was approximately four times higher compared to the other Acinetobacter species (p<0.001). These results suggest that A. indicus-like strains are not cytotoxic which is mainly caused by secreted toxins or virulence factors of the outer membrane. By using knock-out mutants or regulating gene expression previous studies could identify various factors of Acinetobacter species to be involved in either virulence to G. mellonella larvae or cytotoxicity to epithelial cells or both. These factors include the phospholipases C (PLC) [42], phospholipases D (PLD) [43], trimeric autotransporter adhesin Ata [44], acinetobactin outer membrane receptor protein BauA [45], type VI secretion system protein TssM/VasK [46], surface antigen protein 1 (SurA1) [47], RND-type efflux system proteins AdeAB, AdeRS and ArpAB [48, 49], a superoxide dismutase (SodB) [50], and the putative DNA uptake channel protein ComEC [51]. The presence of most of the genes in the genome of A. baumannii strain ATCC 17978 corresponds well with its phenotype in vitro and in vivo, namely high cytotoxicity to epithelial cells and high ability to kill G. mellonella larvae. The absence of several factors, including PLC1 and PLC2, BauA, Ata, AdeRS, and AdeAB in A. lwofii strain ATCC 15309 and the two A. indicus strains might—at least partially—explain the abrogated pathogenicity phenotype in both assays. Although our A. indicus strains and the A. lwofii strain ATCC 15309 strain harboured orthologous genes/proteins of some of these factors, including PLD1-3, TSSM, ArpAB, OmpA, and Omp33, they may have lost their suggested function due to sequence alterations. Complementation of A. indicus strains with virulence factors from A. baumannii and comparative analysis of pathogenicity in vitro and in vivo with the isogenic ancestor will help to identify crucial virulence determinants in future studies.

Taken together, the two A. indicus-like strains IHIT27630 and IHIT27599 showed a comparable pathogenicity with A. lwofii ATCC 15309 which is considered to be a rather low to moderate pathogen. Thus, A. indicus might be considered to be less pathogenic to animals and humans. However, this remains yet unknown for A. indicus-isolates from other sources and should be part of future genomic and functional studies, particularly as this novel species has already been associated with human infection. The fact that cattle are colonized with carbapenem-resistant strains harbouring blaOXA-23 on transposable elements requires further investigations regarding the zoonotic risk of this new Acinetobacter species. One animal which carried the OXA-23 producing A. indicus-like isolate was treated with benzyl-penicillin before, while nothing is known about antibiotic treatment of the other calf. As OXA-23 confers high-level resistance to penicillins and penicillin-β-lactamase inhibitor combinations it can be assumed, that the use of penicillins has created a selective pressure. Even if the suggested pathogenicity of the isolates is rather low, the potential to contribute to the dissemination of the blaOXA-23 gene requires careful consideration. Studies are needed to further explore the cattle population as putative source of carbapenem resistant Acinetobacter spp. strains and of carbapenem resistance determinants to understand spreading of both resistance genes and carbapenem-resistant Acinetobacter species.

Supporting information

S1 Table. Primers and their positions used for mapping of the blaOXA-23 genetic region in bovine A. indicus-like isolates IHIT27599 and IHIT27630.

(DOCX)
S2 Table. SNP matrix referring to S1(A) Fig. Colour shading indicates SNP values (low number of SNPs [dark green] to high number of SNPs [white]). (XLSX)

S3 Table. Median lethal doses (LD50) of Acinetobacter spp. injected into G. mellonella larvae at 24 hours post infection. CI, confidence interval. (DOCX)

S4 Table. LDH statistics. (XLSX)

S1 Fig. Neighbour-joining phylogenetic tree based on (A) the maximum common genome (MCG) of publicly available whole genomes of 32 representative isolates of different Acinetobacter species and (B) the MCG of whole genomes of A. indicus isolates provided publicly and generated in this study. As for the A. indicus type strain A648 three genome sequences under different strain labels (ANC 4215, CIP 110367 and DSM 25388) and accession numbers were available in the database, they were all included in the analysis. The tree was constructed using the maximum likelihood method. Bootstrap values (> 50%) after 1,000 simulations are shown at branch nodes. GenBank accession nos. are given in parenthesis. Bar, 0.05 (A) / 0.01 (B) nucleotide substitutions per site. Results of pairwise distance calculation are provided in S2 Table. (EPS)

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