Abstract: The emergence of multidrug-resistant (MDR) bacterial strains is one of the significant global challenges with regard to bacterial drug-resistance control. Enterobacter hormaechei organisms belong to the Enterobacter cloacae complex (ECC) and are commonly recognized as causative agents for hospital infections. Recently, a few E. hormaechei MDR strains associated with infection in piglets, calves, and a fox were reported, highlighting the important role of animals and livestock in the emergence and spread of antimicrobial resistance. In this study, the vaginal swab sample from a 5-year-old cow with multiple anamnestic infectious abortions was carefully investigated. The animal was unresponsive to antibiotic therapy recommended by the veterinarian. The MDR bacterial strain isolated from the bovine sample, designated as the Saratov_2019, belonged to Enterobacter hormaechei. The genome-based phylogenetic analysis identified the isolate to be Enterobacter hormaechei subsp. xiangfangensis. The genome of the Saratov_2019 contained a 6364 bp plasmid. Importantly, we revealed the novel sequence type ST1416 and 13 MDR genes correlating with the MDR phenotype in only the chromosome but not the plasmid. These findings indicate that the potential spread of this strain may pose a threat for both animal and human health. The data obtained here support the notion of the important role of livestock in the emergence and spread of antimicrobial resistance, promoting careful investigation of the MDR spectra for livestock-related bacterial isolates. To the best of our knowledge, this is the first report on the association of E. hormaechei subsp. xiangfangensis with the infection of the reproductive system in cattle.

Keywords: Enterobacter cloacae complex; Enterobacter hormaechei; multidrug-resistance; MDR; livestock; cattle; MLST; ST

1. Introduction

The emergence and the increase in the finding of multidrug-resistant (MDR) strains is one of the current threats to both human and animal health worldwide [1]. According to the modern classification, MDR strains are non-susceptibility to at least one agent in three or more antimicrobial categories [2]. MDR strains are considered to be the most common causes of morbidity and mortality associated with infectious diseases [3–5]. In fact, certain MDR bacterial strains, which are currently known as ES KAPE organisms (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species) have been included in the WHO global priority pathogens list [6]. These bacterial agents demonstrate resistance to the majority of antibiotics including carbapenems, third-generation cephalosporins, peptide antibiotics, β-lactams, macrolides, and fluoroquinolones, and can cause untreatable severe and often fatal infections such as bloodstream infections and pneumonia. Successful therapy remains challenging and entails the assessment of novel and powerful antibiotics [6].

As members of the ES KAPE group, Enterobacter spp. has been reported to be one of the leading causes of MDR hospital infections [7]. Enterobacter spp. organisms belong to
the family Enterobacteriaceae and are recognized as common causative agents in a variety of bloodstream and intraabdominal infections [8]. Currently, seven species are comprised in the genus Enterobacter (the Enterobacter cloacae complex (ECC)) such as Enterobacter asburiae, Enterobacter carciogenus, Enterobacter cloacae, Enterobacter hormaechei, Enterobacter kobei, Enterobacter nimipressuralis, and Enterobacter mori [9]. Importantly, ECC strains belonging to at least two sequence types (STs), ST78 and ST171, have been recognized as emergent, MDR, and capable of being widespread [10]. Moreover, recently, the E. hormaechei isolate demonstrated a hypervirulent phenotype that was comparable with a hypervirulent K. pneumoniae-type strain in a Galleria mellonella-infection model [11].

Initially, these ESKAPE pathogens were associated with the most recurrent global cause of hospital infections. However, the emergence of MDR strains in animal husbandry indicates the important role of livestock in the emergence and spread of antimicrobial resistance (AMR) and highlights the necessity of the accurate identification of species, subspecies and spectra of AMR and MDR for outbreak-related isolates [12–14] as part of the global strategy to reduce the risk of emergence, spread, and food-borne transmission of MDR strains [14]. Recently, it was reported that Enterobacteriaceae isolated from farm animals may serve as reservoirs of AMR genes [15]. However, only limited information is available on the MDR isolates derived from either animal husbandry or wild-life animals. In fact, only a few ECC strains, namely E. hormaechei, have recently been identified as the causative agents of uterine infection in a dead fox [16], diarrhea in piglets [17], and respiratory disease in unweaned calves in China [18].

This study aimed to investigate the E. hormaechei subsp. xiangfangensis strain Sara-tov_2019 with MDR to eight groups of tested antibiotics, which was isolated from a cow with a reproductive system infection. Typically, Enterobacter spp. obtained from animals possess limited resistance to carbapenems [19], and to our knowledge, the E. hormaechei subsp. xiangfangensis with such an extensive MDR phenotype has not been previously found in cattle.

2. Materials and Methods

2.1. Specimens from a Cow

The vaginal, blood, and uretus swab samples (n = 3) for the study were obtained in April 2019 from a 5-year-old cow with multiple anamnestic infectious abortions. The vaginal swab was collected from a cow after thoroughly cleaning the vulva. The specimens were submitted to the diagnostic laboratory of the Saratov State Agrarian University in order to identify the possible cause of infection. The animal was kept at a small farm in the Saratov Region, Russia. The accompanying report for these animal specimens described the clinical signs of bovine genital tract inflammation such as mucopurulent or purulent vaginal discharge, fever, decreased milk yield, reduced appetite, tiredness, no sign of estrus, and several miscarriages in the past two years. The veterinarian who treated this cow reported that antibiotic therapy including oxytetracycline and third-generation cephalosporins had failed to improve the animal’s health. Thus, the animal was unresponsive to the antibiotic therapy recommended by the veterinarian. These clinical samples were studied bacteriologically using Endo agar (Becton Dickinson, Heidelberg, Germany), and cultivated at 37 °C in an aerobic environment for 3 days, which resulted in the isolation of the bacterial strain from the vaginal specimen only.

2.2. Determination of AMR Phenotype

The bacterial culture was screened for 12 antibiotics of several groups using the disk diffusion test (DDT) [20]. The test-panel of antibiotics included penicillins (Amoxicillin/Clavulanic acid; Pfizer, Inc. USA; Amoxicillin Trihydrate/Colistin Sulfate; Trionis, Russia), third-generation cephalosporins (Ceftriaxone; Biocom, Russia), fourth-generation cephalosporins (Cefquinome; Intervet International B.V., Netherlands), third-generation fluoroquinolone (Enrofloxacain; Bayer, Germany), oxyquinoline (Nitroxoline; Biosintez, Russia), tetracyclines (Oxytetracycline; Nita-farm, Russia), first-generation aminoglycoside
(Kanamycin; PJSC “Krasfarma”, Russia), carbapenems (Meropenem; PJSC “Krasfarma”, Russia), lincosamides (Lincomycin; Velpharm, Russia), macrolides (Azithromycin; Beleka, Belarus), and nitroimidazole (Metronidazole; Nita-farm, Russia). Antibiotic sensitivity was interpreted in accordance with the Clinical and Laboratory Standards Institute (CLSI) [20].

2.3. DNA Extraction and Sequencing

The DNA from the bacterial isolate derived from the corresponding vaginal sample was extracted using the DNeasy Blood and Tissue Qiagen Kit (Qiagen, Hilden, Germany), and concentrations were measured with a spectrophotometer (BioRad Laboratories, Redmond, WA, USA). To preliminarily identify the type of microorganism, the isolated DNA from the bacterial culture was amplified by the 16S rRNA method followed by sequencing, as described previously [21]. The whole genome sequencing procedure of the extracted DNA was performed with the help of an Illumina HiSeq 2500 platform (Genoaanalytica, Moscow, Russia, https://www.genoaanalytica.ru/ (accessed on 15 May 2022)) and MinION (Oxford Nanopore Technologies, Oxford, UK). The DNA library preparation for Nanopore sequencing was undertaken with the 1D Genomic DNA by Ligation (SQK-LSK109) protocol (Oxford Nanopore Technologies, Oxford, UK) including DNA end repair, dA-tailing, and DNA clean-up steps. The final DNA library was sequenced using a FLO-MIN-106 R9.4 flow cell and the MinKNOW software (https://nanoporetech.com/community (accessed on 15 May 2022)).

2.4. Bioinformatic Data Processing

The preliminary DNA identification of the bacterial isolate was conducted through an analysis of the 16S rRNA gene amplicon sequencing and the EzBioCloud database (https://www.ezbiocloud.net (accessed on 15 May 2022)) for subspecies-level identification of the relevant microorganism. Hybrid assembly de novo was generated with Unicycler v 0.4.7 (https://github.com/rrwick/Unicycler (accessed on 15 May 2022)). Contig alignments were performed using Mauve software (http://darlinglab.org/mauve/mauve.html (accessed on 15 May 2022)). Primary metagenomic data analysis was carried out with the help of the Metagenomics Analysis Server MG-RAST (https://www.mg-rast.org (accessed on 15 May 2022)). Genome-based classification and identification were carried out with the help of the Type (Strain) Genome Server (https://tygs.dsmz.de/ (accessed on 15 May 2022)). The whole genome de novo assembled sequence data were deposited in the NCBI GenBank as _E. hormaechei_ subsp. _xiangfangensis_ strain Saratov_2019 (Acc. No. JAHFZP000000000.17).

2.5. MLST-Typing

The identification of allele profiles of multiple contigs and multi-locus sequence typing (MLST) was performed using a PubMLST database (https://pubmlst.org/ (accessed on 15 May 2022)). The ‘house-keeping genes’-derived sequences were deposited in the PubMLST database (https://pubmlst.org/ (accessed on 15 May 2022)), with the access number—ST1416 (https://pubmlst.org/bigsdb?page=profileInfo&db=pubmlst_ecloacae_seqdef&scheme_id=1&profile_id=1416 (accessed on 15 May 2022)).

2.6. Determination of the AMR Genotype

The identification of antibiotic-resistance genes was carried out by using the RGI Resistance Gene Identifier (https://card.mcmaster.ca/analyze/rgi (accessed on 15 May 2022)).

2.7. List of Genomes Included in This Study

The following GenBank accession numbers were used for phylogenetic analysis in this study: NZ_JAHFZP000000001.1 (_E. hormaechei_ subsp. _xiangfangensis_ strain Saratov_2019), CP024908.1 (_E. hormaechei_ subsp. _xiangfangensis_ strain OSUKPC4_1), CP023430.1 (_E. hormaechei_ subsp. _xiangfangensis_ strain WCHEX045001), CP023430.1 (_E. hormaechei_ subsp. _xiangfangensis_ strain
3. Results and Discussion

A single vaginal swab yielded a bacterial strain that was identified by the 16S rRNA gene sequence on the EzBioCloud Server (https://www.ezbiocloud.net (accessed on 15 May 2022)). The results showed that the relevant microorganism was related to *Enterobacter hormaechei*. No microorganisms grew on the Endo agar plates from the blood and urethra specimens.

Overall, 67 contigs were assembled after next-generation sequencing (NGS) of the total DNA derived from the Saratov_2019 strain, of which 66 contigs belonged to the chromosomal DNA, and a single contig was identified as a circular plasmid replicon. The Genome BLAST Distance Phylogeny (GBDP) method based on the comparison of the Saratov_2019 strain with the TYGS database currently consisting of a comprehensive collection of 14,927 microbial type-strain genomes revealed that our strain had the highest homology (93.5%, CI 91.6–95.0) and formed a phylogenetic cluster with the reference strains *E. hormaechei* subsp. *xiangfangensis*, but not with the representatives of other subspecies of *Enterobacter* spp. (Figure 1). Notably, the plasmid identified in the strain designated as *E. hormaechei* Saratov_2019 (Acc. No. in NCBI NZ_JAHFZP010000042.1) with the size of 6364 bp showed high homology (98.84%) with the plasmid replicon pECL-90-4 that was recently (September, 2020) found in the strain *E. hormaechei* NJGLYY90-CR from China (Acc. No. in NCBI CP061745.1). The chromosomes of the strains Saratov_2019 and NJGLYY90-CR also demonstrated high homology (93%, CI 90.7–94.8), and these strains were located in close proximity on the phylogenetic tree (Figure 1). These data evidently indicate that the Saratov_2019 strain belongs to *E. hormaechei*.

The annotation of this strain from the *de novo* assembled whole genome, which was generated with the use of the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) showed the presence of more than 4000 coding sequences (CDSs) and 77 pseudo genes as a result of either the frameshift mutations or premature stop codons in the relevant DNA sequences (Figure 2, Table S2).

Importantly, the Saratov-2019 strain had a novel sequence type (ST), ST1416 (Table 1). The relevant allelic profile consisted of the unique combination of the previously known allelic profiles of seven housekeeping genes: *dnaA*, *fusA*, *gyrB*, *leuS*, *pyrG*, *rplB*, and *rpoB* (https://pubmlst.org (accessed on 15 May 2022)).
Figure 1. Phylogenetic analysis of the Saratov_2019 strain with regard to the different reference Enterobacter spp. genomes conducted with the use of the Type (Strain) Genome Server (TYGS, https://tygs.dsmz.de (accessed on 15 May 2022)). The color coding represents Enterobacter species and subspecies belonging to different clusters, G+C content, genome size, and protein count. The numbers on the branches show the distance between the taxa by delta statistics. The detailed information on the strains is presented in Table S1.

Figure 2. Graphic visualization of the E. hormaechei subsp. xiangfangensis Saratov_2019 genome after the automatic contig annotation that was generated based on the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (https://github.com/ncbi/pgap (accessed on 15 May 2022)). The pie chart (green) demonstrates the number of open reading frames detected. The red diagram shows the number of coding regions in the contigs. The inner chart (yellow) shows the number of contigs. The diagram (black color) shows the distribution of the GC-composition for 66 contigs of the strain. The contigs are located in ascending order of their length from the largest to the smallest.
Table 1. Comparison of the allelic profiles of the seven housekeeping gene loci identified in the whole-genome assembly of the *E. hormaechei* subsp. *xiangfangensis* Saratov_2019 strain of ST1416 and *E. hormaechei* subsp. *xiangfangensis* reference strains* based on the phylogenetic analysis.

| Strain ID                          | ST  | Allele | Reference/Source                                      |
|------------------------------------|-----|--------|-------------------------------------------------------|
| *E. hormaechei* subsp. *xiangfangensis* strain Saratov_2019 | 1416 | 46 21 19 44 13\(^a\) | 4 6 | This study |
| Nameless \(^b\)                      | 1348 | 46 21 19 44 45 4 | 6 | https://pubmlst.org (accessed on 15 May 2022) |
| *E. hormaechei* subsp. *xiangfangensis* LMG27195 | 544  | 10 21 9 44 45 4 | 33 | [22] |
| *E. hormaechei* subsp. *xiangfangensis* strain 34399 | 114  | 53 35 20 44 45 4 | 6 | [23] |
| *E. hormaechei* subsp. *xiangfangensis* strain 34978 | 171  | 49 21 19 44 45 12 | 32 | [24] |
| *E. hormaechei* subsp. *xiangfangensis* strain OSUKPC4_L | 171  | 49 21 19 44 45 12 | 32 | GenBank accession number: CP024908.1 |
| *E. hormaechei* subsp. *xiangfangensis* strain OSUVMCKPC4-2 | 171  | 49 21 19 44 45 12 | 32 | GenBank accession number: CP029246.1 |
| *E. hormaechei* subsp. *xiangfangensis* strain UM_CRE-14 | 171  | 49 21 19 44 45 12 | 32 | https://pubmlst.org (accessed on 15 May 2022) |
| *E. hormaechei* subsp. *xiangfangensis* strain WCHEX045001 | 171  | 49 21 19 44 45 12 | 32 | https://pubmlst.org (accessed on 15 May 2022) |
| *E. hormaechei* subsp. *xiangfangensis* strain Ec61 | 451  | 146 21 148 44 99 4 | 6 | [25] |
| *E. hormaechei* strain NJGLYY90-CR | 418  | 53 35 154 44 45 4 | 6 | https://pubmlst.org (accessed on 15 May 2022) |
| *E. hormaechei* strain 1801 \(^c\) | 78   | 8 9 6 9 9 6 | 8 | https://pubmlst.org (accessed on 15 May 2022) |

* The data are available in the PubMLST database (https://pubmlst.org/) (accessed on 15 May 2022); \(^a\) Different alleles of the gene in comparison with those in the Saratov_2019 strain are shown in red; \(^b\) Information about the origin and characteristics of this strain are absent in the PubMLST database (https://pubmlst.org/) (accessed on 15 May 2022); \(^c\) the reference strain of 20 *E. hormaechei* strains of ST78 with identical allele profiles (the strains ID: 359, 362, 363, 372, 379, 380–383, 388, 391, 392, 401, 407–411, 446, and 491) present in the PubMLST database (https://pubmlst.org/) (accessed on 15 May 2022).

In fact, from three to four out of seven alleles of the Saratov_2019 strain were identical to those of the phylogenetically close reference *E. hormaechei* subsp. *xiangfangensis* strains (Figure 3, Table 1). These identical alleles were as follows: (i) *fusA*, *leuS* and *rplB* in the LMG27195 strain of ST54; (ii) *leuS*, *rplB* and *rpoB* in the strain 34399 of ST114; (iii) *fusA*, *gyrB* and *leuS* in the strains 34978, OSUKPC4_L, OSUVMCKPC4-2, UM_CRE-14, and WCHEX045001 of ST171; and (iv) *fusA*, *leuS*, *rplB* and *rpoB* in the strains Ec61 and NJGLYY90-CR of ST418 and ST451. Nevertheless, the *E. hormaechei* strain of ST1348 had an identical MLST profile in six alleles (4, 6, 19, 21, 44, and 46), and differed from Saratov_2019 by a single SNP for the allele 45 at position 48, which displayed a substitution C->T compared with that of allele 13 of Saratov_2019 corresponding to the *pyrG* gene (Figure 3, Table 1).
Unfortunately, no information on the origin or even the name of this ST1348 isolate is available in the database.

Figure 3. Phylogenetic tree of the strain Saratov_2019 ST1416 (labeled in green) based on a concatenate of the seven housekeeping genes dnaA, fusA, gyrB, leuS, pyrG, rplB and rpoB of Enterobacter (https://pubmlst.org (accessed on 15 May 2022)). The tree was constructed using the maximum likelihood method with MEGA 7.0 [27]. Bootstrap values = 100 (were shown at each node).

No identical alleles were found in the Saratov_2019 strain and the reference E. hormaechei strains of ST78 (Table 1), which together with ST171 was also identified as MDR [10]. In fact, ST78 formed a single branch separately from other STs. Both ST1416 and ST171 were found in the similar branch, although in different but closely related clades (Figure 3), meaning that these STs could have a single common ancestor.

To assess the AMR genotype-to-phenotype prediction of the Saratov_2019 strain, we investigated the relevant genomic data by the CARD service predicting drug-resistance genes. In parallel, the isolate was tested by the disk diffusion method to determine its susceptibility to different classes of antibiotics. The presence of the AMR genes for 16 different groups of antibiotics was predicted by the CARD for Saratov_2019 (Table 2).

At least 13 of them were successfully identified in the genome of this strain (Table 2). Interestingly, the Saratov_2019 strain demonstrated phenotypic resistance for the lincosamide group of antibiotics in DDT. However, it was not possible to identify the genetic determinism of resistance to this group of drugs for this strain. According to the CARD database, today, 38 ontology terms are currently known, which are associated with lincosamide resistance. We performed a comparative alignment of all 38 lincosamide resistance genes to identify a homologous sequence in the Saratov_2019 strain chromosome. Unfortunately, none of the annotated CDSs had homology with the relevant genes of the CARD database. Perhaps the absence of lincosamide resistance genes could be explained by the incomplete assembly of the Saratov_2019 strain chromosome. The Saratov_2019 isolate demonstrated the presence of resistance to eight groups of antibiotics used in the diffusion test, indicating the actual MDR phenotype of this strain. No β-lactamase resistance genes or nine colistin resistance mcr gene variants (mcr-1–mcr-9) [24,28–32] were either predicted or detected during the genome annotation of both the chromosome and plasmid of the Saratov_2019 strain, which correlated with the observed sensitivity of this organism to the carbapenems and β-lactam antibiotics including the β-lactamase inhibitors. In fact, in DDT, the Saratov_2019 strain showed sensitivity to at least two groups of β-lactam antibiotics, Cequinome (fourth-generation cephalosporin) and Meropenem (synthetic antibiotic from the group of carbapenems). Thus, the Saratov_2019 strain was not a carbapenemase-producing E. hormaechei unlike the majority of the E. hormaechei spp. clinical isolates obtained from hospital infections worldwide [12,14,18,33–36].
| No. | ARO Term | AMR Gene Family | Drug Group | Gene | Product | Locus_Tag in the Contig | Contig No. | Drug Group | Confirmation by the DDT |
|-----|-----------|-----------------|------------|------|---------|------------------------|------------|------------|------------------------|
| 1   | Escherichia coli | ampC-type beta-lactamase | C | ampH | D-alanyle-D- alaninecarboxypeptidase/ endopeptidase AmpH multidrug efflux transporter | KK501_00085 | 1 | C (third- generation) | - | + |
| 2   | emrR      | major facilitator superfamily (MFS) antibiotic efflux pump | F | emrR | EmrAB transcriptional repressor EmrR 16S rRNA (adenine(1518)-N(6)/adenine(1519)-N(6))-dimethyltransferase RsmA multidrug efflux RND | KK501_07160 | 5 | - | - | + |
| 3   | rsmA      | resistance-nodulation-cell division (RND) antibiotic efflux pump | F, D | rsmA | multidrug efflux RND transporter permease subunit OqxB | KK501_03125 | 2 | - | - | + |
| 4   | adeF      | resistance-nodulation-cell division (RND) antibiotic efflux pump | F, T | oqxB | multidrug efflux RND transporter permease subunit OqxB | KK501_09445 | 7 | - | - | + |
| 5   | oqxA      | resistance-nodulation-cell division (RND) antibiotic efflux pump | F, G, T, D, Nf | oqxB | multidrug/spermidine efflux transporter permease subunit OqxB | KK501_09445 | 7 | T | - | + |
| 6   | Klebsiella pneumoniae kpnE | superfamily (MFS) antibiotic efflux pump major facilitator | M, Ag, T, P, R | mdtJ | SMR transporter subunit MdtJ | KK501_17290 | 19 | First- generation Ag M | - | + + |
| 7   | Klebsiella pneumoniae kpnE | superfamily (MFS) antibiotic efflux pump major facilitator | M, Ag, C, T, P, R | mdtJ | multidrug/spermidine efflux transporter subunit MdtJ | KK501_17290 | 19 | - | - | + |
| 8   | baeR      | resistance-nodulation-cell division (RND) antibiotic efflux pump | Ag, Ac | baeR | two-component system response regulator BaeR | KK501_18050 | 21 | - | - | + |
| 9   | acrD      | resistance-nodulation-cell division (RND) antibiotic efflux pump | Ag | acrD | multidrug efflux RND transporter permease AcrD | KK501_09635 | 7 | - | - | + |
| 10  | msbA      | (ABC) antibiotic efflux pump | Nm | msbA | ATP-binding protein/permease MsbA | KK501_13920 | 13 | Nm | - | + |
| No. | ARO Term | AMR Gene Family | Drug Group | Gene | Product | Locus_Tag in the Contig | Contig No. | Drug Group | Sensitive | Resistant |
|-----|----------|-----------------|------------|------|---------|------------------------|------------|------------|-----------|-----------|
| 11  | acrR     | AcrA/B complex  | O          | acrR | multidrug efflux transporter, transcriptional repressor AcrR | KK501_00590 | 1 | O          | -         | +         |
| 12  | fosA2    | fosfomycin thiol transferase | Ps | fosA | fosfomycin resistance, glutathione transferase | KK501_03840 | 2 | Ps         | -         | +         |
| 13  | Escherichia coli uhpT | antibiotic-resistant UhpT | Ps | uhpT | hexose-6-phosphate:phosphate antiporter | KK501_18665 | 22 | Ps         | -         | +         |

* Based on the annotation added by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP); ** DDT, the disk diffusion test; a ARO, Antibiotic Resistance Ontology terms based on the CARD (https://card.mcmaster.ca (accessed on 15 May 2022)); b Ac, Aminocoumarins; Ag, Aminoglycosides; C, Cephalosporins; D, Diaminopyrimidines; F, Fluoroquinolone; G, Glycylcyclines; L, Lincomycins; M, Macrolides; Nf, Nitrofurans; Nm, Nitroimidazoles; O, Oxyquinolines; P, Peptide antibiotics; Po, Phenicol antibiotics; Ps, Phosphonic antibiotics (Fosfomycins); R, Rifamycins; T, Tetracyclines.
4. Conclusions

According to our results, we report the first case of isolating the *E. hormaechei* subsp. *xiangfangensis* strain from a cow with a reproductive system infection. This MDR strain had a novel ST1416 and showed no carbapenem resistance. Future prospective research is critical to reveal the actual prevalence of MDR microorganisms in animal husbandry worldwide. A certain limitation of our study was that the panel of antibiotics used only the most common veterinary drugs. Additionally, we plan to significantly extend the number and species of animals investigated in our future research. Due to the potential risk to the health of both animals and humans who are professionally employed in animal husbandry, effective control over the spread of both the AMR and MDR bacterial strains has to be implemented.

**Supplementary Materials:** The following supporting information can be downloaded at: [https://www.mdpi.com/article/10.3390/microorganisms10051036/s1](https://www.mdpi.com/article/10.3390/microorganisms10051036/s1), Table S1: Brief characteristics of the *E. hormaechei* subsp. *xiangfangensis* Saratov_2019 after the automatic contig annotation that was generated based on the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). Table S2: Brief characteristics of the *E. hormaechei* subsp. *xiangfangensis* Saratov_2019 after the automatic contig annotation that was generated based on the NCBI Prokaryotic Genome Annotation Pipeline (PGAP).

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**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the State Ethics Committee, Saratov State Medical University named after V.I. Razumovsky, Saratov, Russia (protocol code A-08-30, 2019-08-26).

**Informed Consent Statement:** Informed consent was obtained from the owner of the cow involved in this study.

**Data Availability Statement:** The data presented in this study are available in the open access databases NCBI GenBank and PubMLST.

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**Conflicts of Interest:** The authors declare no conflict of interest.

**References**

1. García, J.; Rodrígues, F.; Castro, F.; Aires, A.; Marques, G.; Saavedra, M.J. Antimicrobial, Antibiofilm, and Antioxidant Properties of Boletus edulis and Neo Boletus luridiformis Against Multidrug-Resistant ESKAPE Pathogens. *Front. Nutr.* 2021, 8, 773346. [CrossRef] [PubMed]

2. Magiorakos, A.-P.; Srinivasan, A.; Carey, R.B.; Carmeli, Y.; Falagas, M.E.; Giske, C.G.; Harbarth, S.; Hindler, J.F.; Kahlmeter, G.; Olsson-Liljequist, B.; et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* 2012, 18, 268–281. [CrossRef] [PubMed]

3. Mathur, P.; Singh, S. Multidrug Resistance in Bacteria: A Serious Patient Safety Challenge for India. *J. Lab. Physicians* 2013, 5, 5–10. [CrossRef] [PubMed]

4. Levin-Reisman, I.; Ronin, I.; Gefen, O.; Braniss, I.; Shores, N.; Balaban, N.Q. Antibiotic tolerance facilitates the evolution of resistance. *Science* 2017, 355, 826–830. [CrossRef]

5. Medina, E.; Pieper, D.H. Tackling Threats and Future Problems of Multidrug-Resistant Bacteria. *Curr. Top. Microbiol. Immunol.* 2016, 398, 3–33. [CrossRef]

6. Tacconelli, E.; Carrara, E.; Savoldi, A.; Harbarth, S.; Mendelson, M.; Monnet, D.L.; Pulcini, C.; Kahlmeter, G.; Kluytmans, J.; Carmeli, Y.; et al. Discovery, research, and development of new antibiotics: The WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect. Dis.* 2018, 18, 318–327. [CrossRef]
7. Liao, W.; Cui, Y.; Quan, J.; Zhao, D.; Han, X.; Shi, Q.; Wang, Q.; Jiang, Y.; Du, X.; Li, X.; et al. High prevalence of colistin resistance and mcr-9/10 genes in Enterobacter spp. in a tertiary hospital over a decade. Int. J. Antimicrob. Agents 2022, 59, 105673. [CrossRef]

8. Brenner, D.J.; McWhorter, A.C.; Kai, A.; Steigervall, A.G.; Farmer, J.J. Enterobacter asburiae sp. nov., a new species found in clinical specimens, and reassignment of Erwinia dissolvens and Erwinia niimipressuralis to the genus Enterobacter as Enterobacter dissolvens comb. nov. and Enterobacter niimipressuralis comb. nov. J. Clin. Microbiol. 1986, 23, 1114–1120. [CrossRef]

9. Davin-Regli, A.; Lavigne, J.-P.; Pagès, J.-M. Enterobacter spp.: Update on Taxonomy, Clinical Aspects, and Emerging Antimicrobial Resistance. Clin. Microbiol. Rev. 2019, 32, e00002-19. [CrossRef]

10. Villa, J.; Carretero, O.; Viedma, E.; Lora-Tamayo, J.; Mingorance, J.; Chaves, F. Emergence of NDM-7-producing multi-drug-resistant Enterobacter hormaechei sequence type ST-78 in Spain: A high-risk international clone. Int. J. Antimicrob. Agents 2018, 53, 533–534. [CrossRef]

11. Wu, L.; Xu, T.; Ji, Y.; Song, J.; Wang, F.; Huang, J.; Zhou, K. Occurrence and Characteristics of Mcr s among Gram-Negative Bacteria Causing Bloodstream Infections of Infant Inpatients between 2006 and 2019 in China. Microbiol. Spectr. 2022, 10, e0193821. [CrossRef] [PubMed]

12. Beyrouthy, R.; Baret, M.; Marion, E.; Dananché, C.; Dauwalder, O.; Robin, F.; Gauthier, L.; Jousset, A.; Dorlet, L.; Guérin, F.; et al. Novel Enterobacter Lineage as Leading Cause of Nosocomial Outbreak Involving Carbapenemase-Producing Strains.Emerg. Infect. Dis. 2018, 24, 1505–1515. [CrossRef] [PubMed]

13. (Biohaz), E.P.O.B.; Koutsoumanis, K.; Allende, A.; Álvarez-Ordóñez, A.; Bolton, D.; Bover-Cid, S.; Chemaly, M.; Davies, R.; De Cesare, A.; Herman, L.; et al. Role played by the environment in the emergence and spread of antimicrobial resistance (AMR) through the food chain. EFS A 2021, 19, e06531. [CrossRef]

14. EMA Committee for Medicinal Products for Veterinary Use (CVM); EFS Panel on Biological Hazards (BIOHAZ); Murphy, D.; Ricci, A.; Ace, Z.; Beechinh, J.G.; Bergendahl, L.; Breathnach, R.; Bures, J.; Duarte Da Silva, J.P.; et al. EMA and EFS A Joint Scientific Opinion on measures to reduce the need to use antimicrobial agents in animal husbandry in the European Union, and the resulting impacts on food safety (RONAFA). EFS A 2017, 15, e04666. [CrossRef]

15. De Almeida, C.C.; Financi, T.P.; Cardozo, M.V.; Piazzauro, L.J.L.; Pereira, N.; Valmorbida, M.K.; Borzi, M.M.; Weiss, B.; De Ávila, F.A. Enterobacteriaceae in calves, cows and milking environment may act as reservoirs of virulence and antimicrobial resistance genes. Food Sci. Technol. 2020, 43, 376–380. [CrossRef]

16. Shan-Shan, W.; Yun-Jia, S.; Xing-Yang, C.; Cheng-Wei, W.; Shan-Shan, G.U.; Xin, Y.; Shuang, X.; Jun-Wei, G.E.; Hong-Yan, C. Isolation, identification and phylogenetic analysis of Enterobacter hormaechei from foxes. Chin. Vet. Sci. 2017, 47, 768–772.

17. Lu-Yao, L.; Liu, M.; Teng, M.; Wang, L.; Zhang, Y.; Liu, B.J.A.S.V.M. Study on the biological characteristics of Enterobacter hormaechei. J. Anim. Sci. Vet. Med. 2017, 36, 1–6.

18. Wang, Z.; Duan, L.; Liu, F.; Hu, Y.; Leng, C.; Kan, Y.; Yao, L.; Shi, H. First report of Enterobacter hormaechei with respiratory disease in calves. BMC Vet. Res. 2020, 16, 1–4. [CrossRef]

19. Harada, K.; Shimizu, T.; Mukai, Y.; Kuwajima, K.; Sato, T.; Kajino, A.; Usui, M.; Tamura, Y.; Kimura, Y.; Miyamoto, T.; et al. Phenotypic and molecular characterization of antimicrobial resistance in Enterobacter spp. isolates from companion animals in Japan. PLoS ONE 2017, 12, e0174178. [CrossRef]

20. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing, 23rd ed.; Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2013; pp. 29–177. ISBN 1-56238-866-5.

21. Mathew, A.; Joseph, I. Characterization of functionally diverse intestinal bacterial flora of Panulirus homarus (Linnaeus, 1758) along the southwest coast of India. J. Mar. Biol. Assoc. India 2019, 61, 38–43. [CrossRef]

22. Akbari, M.; Bakhshi, B.; Peerayeh, S.N. Particular Distribution of Enterobacter cloacae Strains Isolated from Urinary Tract Infection within Clonal Complexes. Iran. Biomed. J. 2015, 19, 45–55. [CrossRef] [PubMed]

23. Daniels, J.B.; Chen, L.; Groots, S.V.; Mollenkopf, D.F.; Mathys, D.A.; Pancholi, P.; Kreiswirth, B.N.; Wittum, T.E. Enterobacter cloacae Complex Sequence Type 171 Isolates Expressing KPC-4 Carbapenemase Recovered from Canine Patients in Ohio. Antimicrob. Agents Chemother. 2018, 62, 1–6. [CrossRef] [PubMed]

24. Chavda, K.D.; Chen, L.; Fouts, D.E.; Sutton, G.; Brinkac, L.; Jenkins, S.G.; Bonomo, R.A.; Adams, M.D.; Kreiswirth, B.N. Comprehensive Genome Analysis of Carbapenemase-Producing Enterobacter spp.: New Insights into Phylogeny, Population Structure, and Resistance Mechanisms. mBio 2016, 7, e02093-16. [CrossRef] [PubMed]

25. Zhou, H.; Zhang, K.; Chen, W.; Chen, J.; Zheng, J.; Liu, C.; Cheng, L.; Zhou, W.; Shen, H.; Cao, X.; et al. Epidemiological characteristics of carbapenem-resistant Enterobacteriaceae collected from 17 hospitals in Nanjing district of China. Antimicrob. Resist. Infec. Control 2020, 9, 15. [CrossRef] [PubMed]

26. Martins, W.M.B.S.; Martins, E.R.; de Andrade, L.K.; Farzana, R.; Walsh, T.R.; Toleman, M.A.; Nogueira, M.C.L.; Gales, A.C. BKC-2, a New BKC Variant Detected in MCR-9.1-Producing Enterobacter hormaechei subsp. xiangfangensis. Antimicrob. Agents Chemother. 2021, 65, 1–6. [CrossRef]

27. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol. Biol. Evol. 2016, 33, 1870–1874. [CrossRef]

28. Ben Khedher, M.; Baron, S.A.; Riziki, T.; Ruimy, R.; Raoult, D.; Diene, S.M.; Rolain, J.-M. Massive analysis of 64,628 bacterial genomes to decipher water reservoir and origin of mobile colistin resistance genes: Is there another role for these enzymes? Sci. Rep. 2020, 10, 5970. [CrossRef]
29. Lascols, C.; Peirano, G.; Hackel, M.; Laupland, K.B.; Pitout, J.D.D. Surveillance and Molecular Epidemiology of Klebsiella pneumoniae Isolates That Produce Carbapenemases: First Report of OXA-48-Like Enzymes in North America. *Antimicrob. Agents Chemother.* 2013, 57, 130–136. [CrossRef]

30. Lutgring, J.D.; Zhu, W.; de Man, T.J.; Avillan, J.J.; Anderson, K.F.; Lonsway, D.R.; Rowe, L.A.; Batra, D.; Rasheed, J.K.; Limbago, B.M. Phenotypic and Genotypic Characterization of *Enterobacteriaceae* Producing Oxacillinase-48–Like Carbapenemases, United States. *Emerg. Infect. Dis.* 2018, 24, 700–709. [CrossRef]

31. Chavda, K.D.; Westblade, L.F.; Satlin, M.J.; Hemmert, A.C.; Castanheira, M.; Jenkins, S.G.; Chen, L.; Kreiswirth, B.N. First Report of *bla* VIM-4 and *mcr*-9 -Coharboring *Enterobacter* Species Isolated from a Pediatric Patient. *mSphere* 2019, 4, e00629-19. [CrossRef]

32. Ai, W.; Zhou, Y.; Wang, B.; Zhan, Q.; Hu, L.; Xu, Y.; Guo, Y.; Wang, L.; Yu, F.; Li, X. First Report of Coexistence of *bla*SFO–1 and *bla*NDM–1 β-Lactamase Genes as Well as Colistin Resistance Gene *mcr*-9 in a Transferrable Plasmid of a Clinical Isolate of *Enterobacter hormaechei*. *Front. Microbiol.* 2021, 12, 676113. [CrossRef] [PubMed]

33. Tzouvelekis, L.S.; Markogiannakis, A.; Psychogiou, M.; Tassios, P.T.; Daikos, G.L. Carbapenemases in *Klebsiella pneumoniae* and Other *Enterobacteriaceae*: An Evolving Crisis of Global Dimensions. *Clin. Microbiol. Rev.* 2012, 25, 682–707. [CrossRef] [PubMed]

34. Mathers, A.J.; Peirano, G.; Pitout, J.D.D. The Role of Epidemic Resistance Plasmids and International High-Risk Clones in the Spread of Multidrug-Resistant *Enterobacteriaceae*. *Clin. Microbiol. Rev.* 2015, 28, 565–591. [CrossRef] [PubMed]

35. Peirano, G.; Matsumura, Y.; Adams, M.D.; Bradford, P.; Motyl, M.; Chen, L.; Kreiswirth, B.N.; Pitout, J.D. Genomic Epidemiology of Global Carbapenemase-Producing *Enterobacter* spp., 2008–2014. *Emerg. Infect. Dis.* 2018, 24, 1010–1019. [CrossRef]

36. Nordmann, P.; Naas, T.; Poirel, L. Global Spread of Carbapenemase-producing *Enterobacteriaceae*. *Emerg. Infect. Dis.* 2011, 17, 1791–1798. [CrossRef]