Correlation between Polymerase Chain Reaction Identification of Iron Acquisition Genes and an Iron-Deficient Incubation Test for Klebsiella pneumoniae Isolates from Bovine Mastitis

Takeshi Tsuka *, Soma Kumashiro, Tsubasa Kihara and Toshiko Iida

Clinical Veterinary Sciences, Joint Department of Veterinary Medicine, Faculty of Agriculture, Tottori University, 4-101 Koyama-Minami, Tottori 680-8553, Japan; uverworld.t.sid.s@gmail.com (S.K.); kokopenables@gmail.com (T.K.); aratalala@gmail.com (T.I.)

* Correspondence: tsuka@tottori-u.ac.jp; Tel.: +81-857-31-5435

Abstract: We investigated the correlation between the polymerase chain reaction (PCR) identification of six virulence genes associated with siderophore activation and the iron-uptake system (iron-acquisition genes; iucA, entB, fepA, ybtS, pns, and kfu) in mastitis-associated Klebsiella pneumoniae (K. pneumoniae). The growth of 37 K. pneumoniae isolates from the milk of cows with mild mastitis reared on Japanese dairy farms between October 2012 and December 2014 was examined by incubation in an iron-deficient medium.

1. Introduction

Iron is one of the essential nutrients required by bacteria, but it is commonly present at an insufficient level for their growth in their vertebrate hosts [1,2]. Bacteria cannot directly utilize ferric ions (Fe³⁺), a poorly soluble substance that is commonly present in the body cavity [3]. Thus, they can be eliminated by effectively preventing iron acquisition, through using endogenous iron chelators, such as transferrin in serum and lactoferrin in secretory fluids such as milk, as they increase immediately in response to bacterial infection [1]. However, gram-negative bacteria can overcome these host defenses by synthesizing siderophores with an extremely high affinity and specificity for binding to Fe³⁺, which is 10 orders of magnitude higher than those of transferrin and lactoferrin [1,4,5]. The iron-acquisition function of siderophores in mastitis-associated Klebsiella pneumoniae (K. pneumoniae), which is superior to that of lactoferrin, facilitates long-term survival within the mammary gland [6]. Klebsiella species can produce enterobactin, a phenolate siderophore, and aerobactin, a hydroxamate siderophore [1,7]. Yersiniabactin is utilized via the yersiniabactin locus on a high-pathogenicity island in few K. pneumoniae strains, whereas the majority of these bacteria can produce enterobactin [5,8]. Coliform bacteria can utilize bovine mastitis-associated siderophores, such as aerobactin, enterobactin, and yersiniabactin, for surviving within the environment and mammary glands and establishing intramammary infection [2,6,9–12]. Enterobactin assists in intramammary infection [6,10,11], aerobactin contributes to increased mastitis severity in cows [2,9,11], and...
yersiniabactin seems to prolong the intramammary survival of coliform bacteria, resulting in the chronicity of bovine mastitis [12].

The acquisition virulence genes are usually identified by genetic analysis, which mainly involves polymerase chain reaction (PCR) [2,5,9,13,14]. For PCR tests, a primer comprising a short, single-stranded DNA sequence specific to various virulence genes is used [5,9,13,14]. The iucABCD gene cluster, which encodes the proteins involved in aerobactin biosynthesis, is commonly detected in hypervirulent K. pneumoniae because it is located on the same virulence plasmid that encodes the rmpA gene along with the iutA gene, which encodes an aerobactin transporter [15–17]. Enterobactin-associated gene clusters present in the K. pneumoniae chromosome, such as entABCDEF and fepABCDG, encode its biosynthesis and the production of transport-mediated proteins, respectively [15]. Yersiniabactin-associated genes, presenting in the yersiniabactin locus within the high-pathogenicity island, include irp1 and irp2 genes, which encode proteins for yersiniabactin biosynthesis; ybt and fyu genes, which encode the transporters required for yersiniabactin secretion; and the psn gene, which encodes the yersiniabactin receptor [5,15,18]. The kfu operon encodes an ABC transport system facilitating Fe^{3+} uptake [10,16]. However, previous PCR-based studies on mastitis-associated K. pneumoniae isolates have not targeted multiple virulence genes associated with the activation of three siderophore types and the kfu iron-uptake system (referred to as iron-acquisition genes throughout this manuscript).

Laboratory tests are frequently used to investigate the various genotypes of coliform bacteria identified using PCR to assess the association between bacterial phenotype and genotype, such as hypermucoviscosity and drug-resistant phenotypes [7,19]. In vitro laboratory tests, such as inoculation, incubation, or culture tests, have previously been used to demonstrate the degree of association with the targeted bacteria [1,3,5,8,14]. Iron-deficient growth media are commonly used in incubation tests involving coliform bacteria and various siderophores to assess their effects on enhancing bacterial survival under iron-restricted conditions [1,3,8]. Additionally, an iron-deficient incubation test has previously been used to evaluate the growth rates of K. pneumoniae with or without iron-acquisition genes. Bovine mammary epithelial cell cultures have previously been used to identify the adhesion activity associated with fimbriae genes for the in vitro assessment of coliform isolates from bovine mastitis samples [20]. However, mastitis-associated coliform bacteria have not been incubated in an iron-deficient medium previously. Thus, the design of this study, using incubation in an iron-deficient medium has not been employed previously for K. pneumoniae isolates from bovine mastitis.

This study aimed to present the clinical states of the animals with mastitis induced by the K. pneumoniae isolates tested, employ PCR to determine the genes involved in virulence, including six iron-acquisition genes in these K pneumoniae isolates, and identify any correlations between the PCR-identified genes and clinical states, as well as between PCR and iron-deficient incubation results. Additionally, previous medical or laboratory reports were examined to determine whether incubating in an iron-deficient medium is a potentially effective in vitro test to estimate the presence of iron-acquisition genes in K. pneumoniae isolates and the degree of their association in facilitating bovine mastitis.

2. Materials and Methods

2.1. Sampling

Thirty-seven abnormal milk samples were obtained between October 2012 and December 2014 from cows with mild mastitis and reared on 18 dairy farms (farms A–R) located in the central area of Tottori prefecture, Japan. Clinical data for the cows included age, days since calving, and parity at the examination of mastitis milk (Table S1). Additionally, if these animals died by the time of follow-up, the time between their examination and death, and the cause of death were recorded (Table S1). In this study, the mastitis milk conditions of 37 samples were evaluated by two methods: (1) bacterial counts were determined by performing appropriate 10-fold dilutions of the specimens and expressed as log_{10} colony-forming units (CFU)/mL [21]. (2) The macroscopic appearance of the samples was
assigned to one of the four grades according to Gurjar’s method. Score 1: normal, white, homogenous milk; score 2: flake or clot formation in the milk; score 3: a change in the milk appearance to become watery; and score 4: serum- or blood-like changes in the milk [22].

*K. pneumoniae* isolated from the milk specimens using MacConkey–inositol–carbenicillin agar was identified using a VITEK-2 XL Microbiology analyzer (BioMérieux, St. Louis, MO, USA) [23]. The isolates were stored in a skim milk medium at −80 °C until genetic analysis and incubation tests were performed.

2.2. Detection and Identification of Virulence Genes

The frozen specimens were initially thawed, and subsequently cultured in a MacConkey medium. Suspensions were prepared according to the McFarland standard 4 by mixing colonies formed on the medium with sterilized distilled water and incubating at 37 °C for one day. Templates were created by subsequent treatment of the suspensions at 100 °C for 10 min. Primes specific to virulence genes were used for simplex PCR sequencing, as previously described (Table S2) [5,9,13,14]. After mixing 1 µL template with 9 µL premix comprising 1 µL forward primer, 1 µL reverse primer, 7 µL nuclease free water, and 10 µL green master mix, the solution was initially subjected to 30 amplification cycles comprising denaturation at 94 °C for 2 min, followed by annealing at 50 °C for 45 s, and extension at 72 °C for 60 s. Subsequently, the final extension step was performed at 72 °C for 7 min. The products were separated by electrophoresis using a 1.5% agarose gel supplemented with 0.5 µg/mL ethidium bromide and TAE buffer at 100 V. The presence of target genes was confirmed when amplicons with expected sizes were detected.

2.3. Incubation Tests

The experiments were performed in a double-blind format in which one individual (S.K.) performed the isolation tests, without knowing the PCR results that had been sequenced by other individuals (T.K., and T.I.). The iron-deficient medium used in this study was designed according to what has been reported in previous studies [24,25]. The basic medium solution comprised 35 mM glucose, 25 mM NH₄Cl, 1.5 mM KCl, 45.2 mM NaCl, and 0.4 mM MgSO₄·7H₂O, supplemented with 0.5 µM CaCl₂·6H₂O, 0.5 µM H₃BO₃, 0.05 µM CoCl₂·6H₂O, 0.05 µM CuSO₄·7H₂O, 0.05 µM ZnSO₄·7H₂O, 0.05 µM MnSO₄·7H₂O, and 0.005 µM (NH₄)₆Mo₇O₂₄·4H₂O. These components were diluted with 66.7 mM sodium phosphate buffer (pH 7.4) and then adjusted using a mixture of Na₂HPO₄ and NaH₂PO₄. The iron-deficient medium was prepared by batch incubating the solution for one day with Chelex 100 ion-exchange resin (Bio-Rad Laboratories, Inc., Hercules, CA, USA) to remove any iron. Iron-half-sufficient and iron-sufficient media were produced by supplementing the iron-deficient medium with 10 and 20 µM FeSO₄·7H₂O, respectively.

*K. pneumoniae* solutions were prepared according to the McFarland standard 3 by mixing the isolates with sterilized distilled water, naturally thawing the frozen specimens, and incubating in a MacConkey medium at 37 °C for one day. *K. pneumoniae* solutions were diluted by mixing 10 µL bacterial solution with 990 µL pre-prepared substrate solutions (one of the three). *K. pneumoniae* isolates were incubated in an iron-sufficient, iron-half-sufficient, or iron-deficient medium at 37 °C for 1, 2, and 3 days. The bacterial counts were measured using the serial dilution technique with 100 µL aliquots collected from each medium daily.

2.4. Statistical Evaluation

2.4.1. Clinical Data and Mastitis Milk Conditions

In this study, *K. pneumoniae* isolates were grouped as KP0, KP1, KP2, KP3, and KP4 based on the number of iron-acquisition genes (0, 1, 2, 3, and ≥4, respectively), as identified by PCR tests. The Kruskal–Wallis test was used for statistical comparison among these KP groups and total values in terms of the clinical data and milk conditions in the 37 mastitis cases.
2.4.2. Comparison between Inoculation Tests and PCR Identification

In this study, the *K. pneumoniae* counts on days 0, 1, 2, and 3 in iron-sufficient and iron-deficient media were designated as KC0_IS, KC1_IS, KC2_IS, and KC3_IS, respectively. One-way analysis of variance (one-way ANOVA) and Kruskal–Wallis tests were used to compare the positive and negative PCR results for iron-acquisition genes (entB, fepA, ybtS, psn, and kfu) with respect to the association with the bacterial counts on incubation day (0–3 days) in the three media. Additionally, a Kruskal–Wallis test was used to compare the bacterial counts in iron-sufficient, iron-half-sufficient, and iron-deficient media on days 0, 1, 2, and 3.

2.4.3. Association between >8 log_{10} CFU/mL KC3_ID and PCR-Positive Combination of Two Iron-Acquisition Genes

In this study, >8 log_{10} CFU/mL KC3_ID was used as the basis for evaluating *K. pneumoniae* survival under iron-deficient conditions as it was identified as the common count on incubation day 3, in an iron-sufficient medium. In this analysis, PCR-positive combinations of two of five iron-acquisition genes (entB, fepA, ybtS, psn, and kfu) were evaluated. The support, confidence, and lift values were analyzed using association analysis, based on a previously reported statistical analysis of the diagnostic role of milk [26]. In this previous statistical method, the degrees of association were estimated to be strong when the lift values were >1, as a minimum positive dependence effect [26]. Additionally, the chi-square test was used to compare the isolates with each PCR-positive combination and total 37 isolates.

2.4.4. Comparison between Incubation Tests and Numbers of Iron-Acquisition Genes According to the PCR Results

This statistical analysis was conducted for each iron-sufficient, iron-half-sufficient, and iron-deficient incubation test. One-way ANOVA or the Kruskal–Wallis test was used to compare the bacterial counts on days 0, 1, 2, and 3 for each KP group (KP0, KP1, KP2, KP3, and KP4). The Kruskal–Wallis test was used to compare the daily bacterial counts for the various KP groups.

The Scheffe and Mann–Whitney U tests were used for post hoc analysis after the one-way ANOVA and Kruskal–Wallis test throughout this study, respectively. A *p*-value of <0.05 was considered statistically significant.

3. Results

3.1. PCR Identification

PCR did not detect *magA* or *rmpA* genes associated with the hypermucoviscosity phenotype, or the K1 and K2 capsular polysaccharide genes in any of the *K. pneumoniae* isolates. In terms of the PCR identification of the six iron-acquisition genes, the *iucA* gene was not detected in any of the 37 *K. pneumoniae* isolates (Table S3). *entB* and *fepA* genes were most commonly detected using PCR (both, 67.6%; Table S3). The average number of PCR-positive iron-acquisition genes in the isolates was 2.6 (Table S3).

3.2. Clinical Data and Mastitis Milk Conditions

The numbers (proportions) of KP0, KP1, KP2, KP3, and KP4 isolates were 6 (16.2%), 6 (16.2%), 14 (37.8%), 6 (16.2%), and 5 (13.5%), respectively (Table 1). The clinical data and milk conditions were not significantly different among the KP groups and the 37 isolates.
Table 1. Comparison of clinical data [mean (standard deviation)] in mastitis cases, and mastitis milk conditions associated with polymerase-chain-reaction (PCR)-positive numbers of iron-acquisition genes in *Klebsiella pneumoniae* isolates.

| KP Group | Number | Age (days) | Days since Calving at Exam | Parity at Exam | Interval (Days) between Exam and Death | Mastitis Milk Conditions |
|----------|--------|------------|---------------------------|----------------|--------------------------------------|-------------------------|
|          | [Proportion] | at Exam | | | | Bacterial Counts (log_{10} CFU/mL) | Score [20] |
| KP0      | 6 [16.2%] | 2009.0 (757.7) | 220.5 (244.7) | 2.8 (1.3) | 872.5 (754.1) | 5.9 (2.2) | 2.5 (1.2) |
| KP1      | 6 [16.2%] | 1776.4 (823.6) | 137.8 (103.5) | 3.2 (2.0) | 596.2 (529.6) | 7.0 (1.5) | 2.0 (0.6) |
| KP2      | 14 [37.8%] | 2056.2 (757.4) | 203.3 (145.1) | 3.6 (1.7) | 408.4 (346.3) | 5.4 (2.1) | 2.3 (1.0) |
| KP3      | 6 [16.2%] | 2251.8 (536.9) | 74.0 (102.5) | 4.6 (1.3) | 632.0 (636.0) | 6.5 (2.3) | 2.6 (1.1) |
| KP4      | 5 [13.5%] | 1809.6 (718.0) | 93.5 (112.3) | 3.0 (1.8) | 893.5 (464.9) | 5.0 (1.3) | 3.3 (1.0) |
| Total    | 37 [100.0%] | 2000.9 (708.1) | 162.3 (156.1) | 3.5 (1.7) | 620.0 (528.2) | 5.9 (2.0) | 2.4 (1.0) |

1 PCR-positive numbers (0, 1, 2, 3, and >4) of iron-acquisition genes in *Klebsiella pneumoniae* isolates (KP0, KP1, KP2, KP3, and KP4, respectively).

3.3. Comparison between Inoculation Tests and PCR Identification

KC1_{IS} was significantly (*p* < 0.05) higher than KC0_{IS} and similar to KC2_{IS} and KC3_{IS}, which is similar to the growth of PCR-positive and PCR-negative *K. pneumoniae* isolates with each iron-acquisition gene in the iron-sufficient medium (Table 2). Moreover, the KC3_{IS} of *entB*-positive, *fepA*-positive, *psn*-positive, and *kfu*-negative *K. pneumoniae* isolates was significantly (*p* < 0.05) higher than their KC1_{IS}. The growth pattern of *entB*-positive *K. pneumoniae* isolates in the iron-deficient medium was similar to that in the iron-sufficient medium. However, the growth pattern of *entB*-negative *K. pneumoniae* isolates in the iron-deficient medium showed that the KC1_{ID} (7.67 ± 1.08 log_{10} CFU/mL) was significantly (*p* < 0.05) higher than the KC0_{ID} (5.59 ± 0.42 log_{10} CFU/mL), KC2_{ID} (6.42 ± 1.13 log_{10} CFU/mL), and KC3_{ID} (5.89 ± 1.60 log_{10} CFU/mL). Thus, the KC3_{ID} was not significantly different from the KC0_{ID}. Compared with the iron-deficient growth patterns between *entB*-negative and *entB*-positive isolates, significant (*p* < 0.05) differences were found in the KC2_{ID} (6.42 ± 1.13 and 7.72 ± 0.65 log_{10} CFU/mL, respectively), and the KC3_{ID} (6.07 ± 1.60 and 7.63 ± 0.94 log_{10} CFU/mL, respectively).

The growth pattern of *ybtS*-positive and *entB*-positive isolates in the iron-deficient medium were similar; thus, the KC3_{ID} of *ybtS*-positive and *ybtS*-negative isolates were significantly (*p* < 0.05) different (8.43 ± 0.18 and 6.90 ± 1.42 log_{10} CFU/mL, respectively). The iron-deficient growth patterns of *fepA*-negative, *ybtS*-negative, *psn*-negative, and *kfu*-negative *K. pneumoniae* isolates indicate a gradual decrease from KC2_{ID} to KC3_{ID} subsequent to the increase between KC0_{ID} and KC1_{ID}. In the *fepA*-negative isolates, the KC1_{ID} (7.56 ± 1.06 log_{10} CFU/mL) was significantly (*p* < 0.05) higher than the KC0_{ID} (5.64 ± 0.39 log_{10} CFU/mL), and KC3_{ID} (6.07 ± 1.60 log_{10} CFU/mL). Additionally, the KC3_{ID} of *fepA*-positive and *fepA*-negative isolates were significantly (*p* < 0.05) different (7.54 ± 1.08 and 6.07 ± 1.60 log_{10} CFU/mL, respectively). In *ybtS*-negative, *psn*-negative, and *kfu*-negative isolates, the KC0_{ID} were significantly (*p* < 0.05) lower than the KC1_{ID}, KC2_{ID}, and KC3_{ID}. The iron-half-sufficient growth patterns tended to be similar to the iron-sufficient growth patterns (Table S4).
Table 2. Association between bacterial counts [means (standard deviations); log\textsubscript{10} colony-forming units/mL] and polymerase-chain-reaction (PCR)-positive iron-acquisition genes in \textit{Klebsiella pneumoniae} isolates incubated in iron-sufficient and iron-deficient medium.

| Gene | PCR | Iron-Sufficient Incubation | Iron-Deficient Incubation |
|------|-----|-----------------------------|---------------------------|
|      |     | Day 0 | Day 1 | Day 2 | Day 3 | Day 0 | Day 1 | Day 2 | Day 3 |
|      |     |       |       |       |       |       |       |       |       |
| \textit{entB} | + | 5.44 (0.26) \textsuperscript{a} | 7.73 (1.00) \textsuperscript{b,c} | 8.30 (0.65) \textsuperscript{b,d} | 8.37 (0.70) \textsuperscript{b,d} | 5.45 (0.17) \textsuperscript{a} | 7.42 (0.93) \textsuperscript{b} | 7.72 (0.65) \textsuperscript{b,e} | 7.63 (0.94) \textsuperscript{b,e} |
| \textit{entB} | − | 5.50 (0.20) \textsuperscript{a} | 7.74 (1.10) \textsuperscript{b} | 7.93 (0.63) \textsuperscript{b} | 8.08 (0.61) \textsuperscript{b} | 5.59 (0.42) \textsuperscript{a} | 7.67 (1.08) \textsuperscript{b,c} | 6.42 (1.13) \textsuperscript{b,d,f} | 5.89 (1.60) \textsuperscript{d,g} |
| \textit{fepA} | + | 5.44 (0.26) \textsuperscript{a} | 7.75 (0.97) \textsuperscript{b,c} | 8.23 (0.66) \textsuperscript{b} | 8.34 (0.71) \textsuperscript{b,d} | 5.42 (0.17) \textsuperscript{a} | 7.47 (0.96) \textsuperscript{b} | 7.39 (1.02) \textsuperscript{b} | 7.54 (1.08) \textsuperscript{b,e} |
| \textit{fepA} | − | 5.50 (0.20) \textsuperscript{a} | 7.68 (1.17) \textsuperscript{b} | 8.07 (0.65) \textsuperscript{b} | 8.14 (0.62) \textsuperscript{b} | 5.64 (0.39) \textsuperscript{a} | 7.56 (1.06) \textsuperscript{b,c} | 7.04 (1.09) \textsuperscript{b} | 6.07 (1.60) \textsuperscript{b,d,f} |
| \textit{ybtS} | + | 5.41 (0.33) \textsuperscript{a} | 7.58 (1.32) \textsuperscript{b} | 8.53 (0.34) \textsuperscript{b} | 8.70 (0.22) \textsuperscript{b} | 5.38 (0.25) \textsuperscript{a} | 7.81 (0.63) \textsuperscript{b} | 8.20 (0.57) \textsuperscript{b} | 8.43 (0.18) \textsuperscript{b,e,g} |
| \textit{ybtS} | − | 5.47 (0.24) \textsuperscript{a} | 7.75 (1.00) \textsuperscript{b} | 8.14 (0.68) \textsuperscript{b} | 8.22 (0.70) \textsuperscript{b} | 5.51 (0.28) \textsuperscript{a} | 7.47 (1.00) \textsuperscript{b} | 7.16 (1.03) \textsuperscript{b} | 6.90 (1.42) \textsuperscript{b,f} |
| \textit{psn} | + | 5.47 (0.24) \textsuperscript{a} | 7.89 (1.08) \textsuperscript{b,c} | 8.13 (0.82) \textsuperscript{b} | 8.28 (0.74) \textsuperscript{b,d} | 5.46 (0.20) \textsuperscript{a} | 7.66 (0.92) \textsuperscript{b} | 7.84 (0.79) \textsuperscript{b} | 7.35 (1.38) \textsuperscript{b} |
| \textit{psn} | − | 5.46 (0.25) \textsuperscript{a} | 7.68 (1.01) \textsuperscript{b} | 8.19 (0.62) \textsuperscript{b} | 8.27 (0.68) \textsuperscript{b} | 5.51 (0.30) \textsuperscript{a} | 7.46 (1.00) \textsuperscript{b} | 7.14 (1.05) \textsuperscript{b} | 6.99 (1.45) \textsuperscript{b} |
| \textit{kfu} | + | 5.37 (0.20) \textsuperscript{a} | 7.99 (0.87) \textsuperscript{b} | 8.15 (0.84) \textsuperscript{b} | 8.18 (0.83) \textsuperscript{b} | 5.47 (0.18) \textsuperscript{a} | 7.83 (1.04) \textsuperscript{b} | 7.64 (0.80) \textsuperscript{b} | 7.78 (0.89) \textsuperscript{b} |
| \textit{kfu} | − | 5.51 (0.26) \textsuperscript{a} | 7.58 (1.08) \textsuperscript{b,c} | 8.20 (0.55) \textsuperscript{b} | 8.32 (0.60) \textsuperscript{b,d} | 5.51 (0.32) \textsuperscript{a} | 7.33 (0.92) \textsuperscript{b} | 7.06 (1.11) \textsuperscript{b} | 6.68 (1.53) \textsuperscript{b} |
| Total | | 5.46 (0.24) \textsuperscript{a} | 7.73 (1.02) \textsuperscript{b,c} | 8.18 (0.66) \textsuperscript{b} | 8.27 (0.68) \textsuperscript{b,d} | 5.49 (0.28) \textsuperscript{a} | 7.50 (0.98) \textsuperscript{b} | 7.28 (1.04) \textsuperscript{b} | 7.07 (1.43) \textsuperscript{b,h} |

\textsuperscript{1} + and −. Each iron-acquisition gene was detected or not detected by the PCR test, respectively. \textsuperscript{a,b,c,d} Within row (among 0–3 incubation days) for each incubation group, numbers with different superscripts are significantly different (\(p < 0.05\)). \textsuperscript{e,f} Within column (between PCR\textsuperscript{+} and PCR\textsuperscript{−}) for each iron-acquisition gene group, numbers with different superscripts are significantly different (\(p < 0.05\)). \textsuperscript{g,h} Within column between each iron-acquisition gene group and total values, numbers with different superscripts are significantly different (\(p < 0.05\)).
3.4. Association between the KC3\textsubscript{ID} of >8 log\textsubscript{10} CFU/mL and PCR-Positive Combination of Two Iron-Acquisition Genes

The most common combination of two PCR-positive iron-acquisition genes related to >8 log\textsubscript{10} CFU/mL KC3\textsubscript{ID} comprised entB and fepA genes (support value: 0.30), but the lift value (1.02) was close to 1 (implying a minimum positive dependence effect; Table 3). The combination of PCR-positive ybtS and kfu genes contributed to >8 log\textsubscript{10} CFU/mL KC3\textsubscript{ID}, with the highest lift value (12.33) but the lowest support value (0.08). In this analysis, many \textit{K. pneumoniae} isolates with the PCR-positive ybtS gene, as one of two iron-acquisition genes, could be grown at >8 log\textsubscript{10} CFU/mL in the iron-deficient medium within three days (Table 3).

Table 3. Proportion (number) of polymerase-chain-reaction (PCR)-positive combinations of two iron-acquisition genes, and the association analysis \(^1\) between the combination and the measurements of \textit{Klebsiella pneumoniae} counts of >8 log\textsubscript{10} colony-forming units/mL on three incubation days in iron-deficient medium (basic KC3\textsubscript{ID}).

| Combination of Two Genes Listed within Column and Row | fepA          | ybtS          | psn          | kfu          |
|------------------------------------------------------|---------------|---------------|--------------|--------------|
| entB                                                 | 54.1% (\(n = 20\)) S: 0.30, C: 0.35, L: 1.02 | 10.8% (\(n = 4\)) S: 0.11, C: 1.00, L: 9.25 \(*\) | 18.9% (\(n = 7\)) S: 0.11, C: 0.37, L: 3.02 | 29.7% (\(n = 11\)) S: 0.19, C: 0.64, L: 2.14 |
| fepA                                                 | -             | 10.8% (\(n = 4\)) S: 0.11, C: 1.00, L: 9.25 \(*\) | 13.5% (\(n = 5\)) S: 0.11, C: 0.80, L: 5.92 \(*\) | 29.7% (\(n = 11\)) S: 0.16, C: 0.55, L: 1.83 |
| ybtS                                                 | -             | -             | 10.8% (\(n = 4\)) S: 0.11, C: 1.00, L: 9.25 \(*\) | 8.1% (\(n = 3\)) S: 0.08, C: 1.00, L: 12.33 \(*\) |
| psn                                                  | -             | -             | -             | 13.5% (\(n = 5\)) S: 0.08, C: 0.60, L: 4.44 |

\(^1\) Support, confidence, and lift values are abbreviated as “S”, “C”, and “L”, respectively, in this table. \(*\) Associations between PCR-positive combinations of two genes for the achievement of basic KC3\textsubscript{ID} are significant (\(p < 0.05\)) using chi-square test.

3.5. Comparison between Incubation Tests and PCR-Positive Numbers of Iron-Acquisition Genes

The growth patterns of KP1, KP2, KP3, and KP4 in iron-sufficient medium for 0–3 days were similar, and the KC0\textsubscript{IS} of all KP groups were significantly (\(p < 0.05\)) lower than their KC1\textsubscript{IS}, KC2\textsubscript{IS}, and KC3\textsubscript{IS}, except there was no significant difference between KC0\textsubscript{IS} and KC1\textsubscript{IS} in KP0 (Figure 1).

The KC1\textsubscript{ID}, KC2\textsubscript{ID}, and KC3\textsubscript{ID} of KP2, KP3, and KP4 isolates were significantly (\(p < 0.05\)) increased compared to their KC0\textsubscript{ID} (Figure 2). The values tended to be higher in an order dependent on the number of PCR-positive iron-acquisition genes in KC1\textsubscript{ID}, KC2\textsubscript{ID}, and KC3\textsubscript{ID}. The KC1\textsubscript{ID} of KP0 isolates were significantly (\(p < 0.05\)) higher than their KC0\textsubscript{ID}, followed by gradual decreases in KC2\textsubscript{ID} and KC3\textsubscript{ID}. However, the KC2\textsubscript{ID} and KC3\textsubscript{ID} of KP0 isolates were not significantly different from their KC0\textsubscript{ID}. Interestingly, the KC2\textsubscript{ID} of KP0 and KP4 isolates were significantly (\(p < 0.05\)) different. Furthermore, the KC3\textsubscript{ID} of KP0 isolates was significantly (\(p < 0.05\)) lower than that of KP2, KP3, and KP4 isolates. The growth pattern of KP1 isolates was similar to that of KP0 isolates, where the significant (\(p < 0.05\)) increase between KC0\textsubscript{ID} and KC1\textsubscript{ID} was followed by a gradual decrease from KC1\textsubscript{ID} to KC3\textsubscript{ID}. Interestingly, the KC3\textsubscript{ID} of KP1 isolates was significantly (\(p < 0.05\)) lower than that of KP4 isolates. The growth patterns associated with the variation in number of PCR-positive iron-acquisition genes in iron-half-sufficient medium were similar to those in the iron-sufficient medium (Figure S1).
Microorganisms 2022, 10, 1138

Figure 1. Correlations among *Klebsiella pneumoniae* isolates grouped by KP0 (white), KP1 (diagonal), KP2 (gray), KP3 (dotted), and KP4 (black) for the growths in iron-sufficient medium. * Asterisks denote significant ($p < 0.05$) differences for each value of KP groups on incubation day 0.

Figure 2. Correlations among *Klebsiella pneumoniae* isolates grouped by KP0 (white), KP1 (diagonal), KP2 (gray), KP3 (dotted), and KP4 (black) for the growths in iron-deficient medium. * Asterisks denote significant ($p < 0.05$) differences for each value of KP groups on incubation day 0. † Dagger symbols denote significant ($p < 0.05$) differences between KP groups, connected by a line.
4. Discussion

This study identified the variations in the number of PCR-positive iron-acquisition genes in K. pneumoniae isolates between farms. Several genotypes of mastitis-associated K. pneumoniae have frequently been detected [27,28], because multiple genotypes of this pathogen are commonly present in sawdust bedding and the feces of cows reared in each farm [6]. The extended intra-farm distribution of K. pneumoniae might have contributed to the variety of PCR-positive iron-acquisition genes in this study. The average age and parity of the 37 cows used in this study were 5.5 years and 3.5, respectively, with the average mastitis duration being 162 days after calving. These data are similar to previously reported findings, where mild mastitis occurred during 146 lactation days in 4.1 years-old cows with 2.7 average parity [21]. Additionally, the prevalence of Klebsiella-associated mastitis has previously accounted for approximately 30% at >100 days after calving, although approximately 50% at <30 days after calving [29]. The bacterial counts in the milk of cows with mild mastitis in this study were 5.9 log10 CFU/mL, which is within the previously identified level (>5.0 log10 CFU/mL) in >25% of mild mastitis milks, although previous reports have also shown that 6.0 log10 CFU/mL develops acute clinical signs of coliform mastitis [21,29]. However, the clinical data of K. pneumoniae-infected cows with mastitis were not related to K. pneumoniae identification using PCR.

PCR analysis of 37 K. pneumoniae isolates did not detect the rmpA and magA genes related to the hypermucoviscosity phenotype and the iucA gene, which is related to aerobactin biosynthesis and found on the same virulence plasmid, in any isolate [9,15–17]. Hypervirulent K. pneumoniae strains with the virulence plasmid encoding these genes exhibit predominant aerobactin production-associated siderophore activity, in contrast to the reduced enterobactin and yersiniabactin-associated activity [15–17,19]. Previous PCR analyses of K. pneumoniae isolated from mastitis milk found that the prevalence of iucA-positive isolates ranged between 66.7% and 100% [2,9]. Additionally, the hypermucoviscosity phenotype associated with rmpA and magA expression was found in 16% of K. pneumoniae isolates from bovine mastitis samples [16]. In contrast, the PCR-negative K. pneumoniae isolates for the iucA, rmpA, and magA genes in this study seemed to belong to the classical K. pneumoniae strains, which distinguished them from hypervirulent K. pneumoniae strains [7]. Therefore, classical K. pneumoniae strains may have been one of the possible causes of the present bovine cases, as they involved mild mastitis. A previous study has predominantly detected aerobactin in K. pneumoniae isolates from animals with moderate to severe clinical mastitis using PCR [11].

Interestingly, the growth pattern of K. pneumoniae isolates in the iron-deficient medium was identical to the common patterns in iron-sufficient incubation, even though these isolates were PCR-positive for only one of the six iron-acquisition genes analyzed in this study. In particular, the growth of entB-positive isolates in the iron-deficient medium was significantly enhanced between days 2 and 3. Enterobactin, which is biosynthesized by an entB gene-encoded protein, is one of the most common siderophores secreted by K. pneumoniae when it infects the mammary glands, facilitating increased intramammary colonization [6,10,11]. The KC3ID of isolates that were PCR-positive and PCR-negative for ybtS and fepA were also significantly different. Yersiniabactin, which is biosynthesized by a ybtS gene-encoded protein, may promote chronicity of bovine mastitis via prolonged survival within infected mammary glands because of its function in biofilm formation as well as the iron-acquisition system in iron-deficient environments [12]. Mastitis-associated coliform bacteria predominantly possess the fepA gene, the activity of which is necessary for interacting with the enterobactin-mediated iron retrieval system on the cellular surface [6]. The kfu- and psn-positive K. pneumoniae strains seemed to minimize the iron-deficient-associated decrease between KC2ID and KC3ID. The association of the psn gene with bovine mastitis is not well known, because there are no previous reports about this association, while the kfu iron-acquisition system is assumed to play a common role in promoting intramammary infection by mastitis-associated K. pneumoniae; the prevalence of kfu-positive K. pneumoniae accounts for 25% of caprine mastitis and 77.8% of bovine and
buffer mastitis [2,13,16]. Additionally, the kfu iron-acquisition system may enhance bovine mastitis severity based on the association of kfu genes with subclinical and clinical bovine mastitis prevalence (20% vs. 39%, respectively) [10].

In addition to evaluating single iron-acquisition genes, assessing the associations between the number of PCR-positive genes and combinations of multiple iron-acquisition genes from the results of iron-deficiency incubation tests is useful because interactions between various iron-acquisition systems may enhance the bacterial potential to adapt to iron-deficient environments [2,7,8,14,17]. Accordingly, the combination of entB and kepA genes was most commonly associated with >8 log_{10} CFU/mL KC3_{ID}. In mastitis-associated K. pneumoniae isolated in our field study, the enterobactin-mediated iron-acquisition system enhanced by the yersiniabactin-mediated system may contribute to prolonged survival within the iron-deficient conditions of infected mammary glands [2,14,15]. Additionally, entB gene expression followed by ybtS gene expression promotes siderophore activation in 46.3–83.7% K. pneumoniae isolates with these genes [17]. However, the highest association of this combination with this criterion was simply caused by the highest PCR-positive proportion of this combination (54.1%) compared to those of the other combinations (8.1–29.7%). Based on the lift values, the combination of the ybtS gene with the other genes had high contributions for this criterion despite their low PCR-positivity (8.1–13.5%). The previous PCR analysis for classical K. pneumoniae strains related to human infectious diseases identified that the PCR-positivity of the ybtS gene was lower than that of the entB gene [30]. Co-activating the genes encoding siderophore transport or receptor systems, together with their biosynthesis genes, such as entB and ybtS, may influence the enhanced pathogenicity of some strains [8]. Co-expression of ybt and psl genes may be induced in K. pneumoniae with a high-pathogenicity island locus encoding these genes under iron-deficient conditions [5,15,18]. However, no previous reports have focused on the role of enhanced yersiniabactin-mediated pathogenicity resulting from co-activation of the ybtS and psl genes associated with bovine mastitis. The increased association of the combination of ybtS and kfu genes with bovine mastitis may be supported by previous inoculation tests identifying the significant effects of decreasing the lethal doses in mice infected with K. pneumoniae strains with these two genes, possibly facilitating their growth in host animals [8].

All five KP4 isolates in this study could utilize enterobactin and yersiniabactin, and four of the five isolates might have the kfu iron-acquisition system. K. pneumoniae strains with multiple iron-acquisition systems account for >90% of pathogenic K. pneumoniae strains in humans [17]. Virulence genes encoding enterobactin, aerobactin, and yersiniabactin have also frequently been detected in PCR analyses of K. pneumoniae isolates from environmental samples collected from dairy farms, as well as mastitis milk samples [2,11,20]. Coliform bacteria, including K. pneumoniae, can profitably utilize several iron chelators when invading and subsequently surviving within the mammary glands due to the selective and effective diversion of these acquired substances for their survival in the dairy environment [2]. Intramammary infections caused by these bacteria can facilitate frequent occurrences of mild or persistent mastitis with acute clinical signs [2,31,32]. Interestingly, no predominant virulence gene combination impacts the severity of Escherichia coli-associated mastitis [33]. The relationship of our data with the severity of K. pneumoniae-associated mastitis should be further assessed, because isolates from mild mastitis cases were targeted in this study.

Our study highlighted the utilization of iron-deficient incubation to assess correlations with PCR identification. Time-dependent changes in the incubation tests appeared to differ from those reported in a previous study, showing a sharp increase in K. pneumoniae counts (6–8 log_{10} CFU/mL) within 6 h, followed by a plateau at the level of 8 log_{10} CFU/mL between 6 and 24 h [4]. A previous study has reported that K. pneumoniae with various iron-acquisition genes grow rapidly up to 8 h, followed by a gradual increase between 8 and 24 h, regardless of the intra-medium iron concentrations (0, 10, 30, and 50 μM), corresponding to the levels between the iron-deficient and iron-sufficient media used in this study [3]. Contrary to previous growth changes within 24 h [3,4], our preliminary experiments have reported the continuous, rapidly increasing growth of K. pneumoniae
incubated in an iron-sufficient medium for 24 h (data not shown). During this incubation period, *K. pneumoniae* may utilize Fe\(^{3+}\) ions stored within the cells, regardless of the genes identified by PCR. Based on the preliminary tests, incubation tests were designed to be performed over three days. The results on the third day enabled us to identify the differences among variations in the PCR identifications of iron-acquisition genes. On the third day of incubation, the counts of *K. pneumoniae* with no PCR-detected iron-acquisition genes might have naturally decreased, because the death rates exceeded their growth rates due to Fe\(^{3+}\) ion depletion. *K. pneumoniae* may be more efficient for acquiring poor Fe\(^{3+}\) ion concentration, dependent upon the increased number of PCR-positive iron-acquisition genes. The iron concentration in the iron-deficient medium described previously and used in this study is less than 0.5 µM \[3,25\]. Our study using iron-deficient incubation tests can be developed by including other laboratory tests, such as bovine mammary epithelial cell culture to identify bacterial adhesion function, for the further investigation of the virulence genes facilitating *K. pneumoniae*-associated bovine mastitis \[20\].

5. Conclusions

In this study, PCR analysis of six iron-acquisition genes (*iucA*, *entB*, *fepA*, *ybtS*, *psn*, and *kfu*) using 37 *K. pneumoniae* isolates from bovine mastitis milk confirmed the higher proportions of PCR detection for *entB* and *fepA* genes, contrary to the lack of PCR detection for the *iucA* gene. The growth pattern of *K. pneumoniae* isolates that were PCR-positive for each iron-acquisition gene in the iron-deficient medium were identical to those in the iron-sufficient medium. The count of mastitis-associated *K. pneumoniae* isolates that were PCR-positive for *ybtS* and the other iron-acquisition genes (*entB*, *fepA*, *psn*, and *kfu*) incubated in iron-deficient and iron-sufficient media were mostly >8 log\(_{10}\) CFU/mL, as a level of three-day incubation. Moreover, *K. pneumoniae* isolates with four and five PCR-positive iron-acquisition genes could grow in the iron-deficient medium for three days compared to those with 0 and 1 PCR-positive iron-acquisition genes. This iron-deficient incubation test is so simple that it is routinely applicable in laboratories in bovine practice, which are not commonly equipped with advanced examination devices (such as a PCR analyzer). Evaluating iron-deficient incubation growth using 8 log\(_{10}\) CFU/mL KC31D may contribute to estimating the degree of iron-acquisition function in mastitis-associated *K. pneumoniae* the need to perform PCR analysis.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms10061138/s1, Table S1: Clinical data and milk conditions in bovine cases of mild mastitis caused by intramammary infections with *Klebsiella pneumoniae* tested in this study; Table S2: Primers used in this study; Table S3: Polymerase chain reaction (PCR) detection of six iron-acquisition genes in *Klebsiella pneumoniae* isolates tested in this study; Table S4: Association between bacterial counts [means (standard deviations); log\(_{10}\) colony-forming units/mL] and polymerase-chain-reaction (PCR)-positive iron-acquisition genes in *Klebsiella pneumoniae* isolates incubated in iron-half-sufficient medium; Figure S1: Correlations among *Klebsiella pneumoniae* isolates grouped by KP0 (white), KP1 (diagonal), KP2 (gray), KP3 (dotted), and KP4 (black) for the growths in iron-half-sufficient medium. Asterisks denote significant (\(p < 0.05\)) differences for each value of KP groups on incubation day 0.

Author Contributions: Conceptualization, T.T.; methodology, T.T.; validation, S.K., T.K. and T.I.; investigation, T.T., S.K., T.K. and T.I.; data curation, T.T.; writing—original draft preparation, T.T.; writing—review and editing, T.T., S.K., T.K. and T.I.; supervision, T.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data used to support the findings of this study are available from the corresponding author upon request.
Acknowledgments: We thank our colleagues in Tottori University for helpful the technical support.

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

References

1. Williams, P.; Chart, H.; Griffiths, E.; Stevenson, P. Expression of high affinity iron uptake systems by clinical isolates of Klebsiella. *FEMS Microbiol. Lett.* 1987, 44, 407–412. [CrossRef]

2. Paulin-Curlee, G.G. Characterization of virulence-related genes in Klebsiella pneumoniae from dairy herds. In *Mastitis Associated Klebsiella pneumoniae Isolates Show High Levels of Genetic Isolates*; Paulin-Curlee, G.G., Ed.; ProQuest: Ann Arbor, MI, USA, 2007; pp. 91–112.

3. Chen, T.; Dong, G.; Zhang, S.; Zhang, X.; Zhao, Y.; Cao, J.; Zhou, T.; Wu, Q. Effects of iron on the growth, biofilm formation and virulence of Klebsiella pneumoniae causing liver abscess. *BMC Microbiol.* 2020, 20, 36. [CrossRef]

4. Perry, R.D.; San Clemente, C.L. Siderophore synthesis in Klebsiella pneumoniae and Shigella sonnei during iron deficiency. *J. Bacteriol.* 1979, 140, 1129–1132. [CrossRef][PubMed]

5. Lawlor, M.S.; O’Connor, C.; Miller, V.L. Yersiniabactin is a virulence factor for Klebsiella pneumoniae during pulmonary infection. *Infect. Immun.* 2007, 75, 1463–1472. [CrossRef][PubMed]

6. Hogan, J.; Smith, K. Coliform mastitis. *Vet. Res.* 1984, 34, 507–519. [CrossRef]

7. Guin, T.A.; Olson, R.; Macdonald, U.; Metzger, D.; Malteese, L.M.; Drake, E.J.; Gulick, A.M. Aerobactin mediates virulence and accounts for increased siderophore production under iron-limiting conditions by hypervirulent (hypermucoviscous) Klebsiella pneumoniae. *Infect. Immun.* 2014, 82, 2356–2367. [CrossRef]

8. Hsieh, P.F.; Lin, T.L.; Lee, C.Z.; Tsai, S.F.; Wang, J.T. Serum-induced iron-acquisition systems and tonB contribute to virulence in Klebsiella pneumoniae causing primary pyogenic liver abscesses. *J. Infect. Dis.* 2008, 197, 1717–1727. [CrossRef]

9. Osman, K.M.; Hassan, H.M.; Orabi, A.; Abdelhafez, A.S.T. Phenotypic, antimicrobial susceptibility profile and virulence factors of Klebsiella pneumoniae isolated from buffalo and cow mastitic milk. *Pathog. Glob. Health* 2014, 108, 191–199. [CrossRef]

10. Yang, Y.; Higgins, C.H.; Rehan, I.; Galvao, K.N.; Brito, I.L.; Bicalho, M.L.; Song, J.; Wang, H.; Bicalho, R.C. Genetic diversity, virulence, and antimicrobial resistance of Klebsiella pneumoniae strains from cows and humans. *Appl. Environ. Microbiol.* 2019, 85, e02654-18. [CrossRef]

11. Cheng, J.; Zhou, M.; Nobrega, D.B.; Cao, Z.; Yang, J.; Zhu, C.; Han, B.; Gao, J. Virulence profiles of Klebsiella pneumoniae isolated from 2 large dairy farms in China. *J. Dairy Sci.* 2021, 104, 9027–9036. [CrossRef]

12. Blum, S.E.; Heller, E.D.; Sela, S.; Elad, D.; Edery, N.; Leitner, G. Genomic and phenomic study of mammary pathogenic *Escherichia coli*. *PLoS ONE* 2015, 10, e0136387.

13. Aher, T.; Roy, A.; Kumar, P. Molecular detection of virulence genes associated with pathogenicity of Klebsiella spp. isolated from the respiratory tract of apparently healthy as well as sick goats. *Israel J. Vet. Med.* 2012, 67, 249–252.

14. Bachman, M.A.; Oyler, J.E.; Burns, S.H.; Caza, M.; Lepine, F.; Dozois, C.M.; Weiser, J.N. Klebsiella pneumoniae yersiniabactin promotes respiratory tract infection through evasion of lipocalin. *Infect. Immun.* 2011, 79, 3309–3316. [CrossRef][PubMed]

15. Paczosa, M.K.; Mecsas, J. Klebsiella pneumoniae: Going on the offense with a strong defense. *Microbiol. Mol. Biol. Rev.* 2016, 80, 629–661. [CrossRef]

16. Gao, J.; Li, S.; Zhang, J.; Zhou, Y.; Xu, S.; Barkema, H.W.; Nobrega, D.B.; Zhu, C.; Han, B. Prevalence of potential virulence genes in Klebsiella spp. Isolated from cows with clinical mastitis on large Chinese dairy farms. *Foodborne Pathog. Dis.* 2019, 16, 856–863. [CrossRef]

17. Remya, P.A.; Shanthi, M.; Sekar, U. Characterisation of virulence genes associated with pathogenicity in *Klebsiella pneumoniae*. *Indian J. Med. Microbiol.* 2019, 37, 210–218. [CrossRef]

18. Bach, S.; de Almeida, A.; Carniel, E. The *Yersinia* high-pathogenicity island is present in different members of the family *Enterobacteriaceae*. *FEMS Microbiol. Lett.* 2000, 183, 289–294. [CrossRef]

19. Russo, T.A.; Olson, R.; MacDonald, U.; Beanan, J.; Davidson, B.A. Aerobactin, but not yersiniabactin, salmochelin, or enterobactin, enables the growth/survival of hypervirulent (hypermucoviscous) *Klebsiella pneumoniae* ex vivo and in vivo. *Infect. Immun.* 2015, 83, 3325–3333. [CrossRef]

20. Cheng, J.; Zhang, J.; Yang, J.; Yi, B.; Liu, G.; Zhou, M.; Kastelic, J.P.; Han, B.; Gao, J. Klebsiella pneumoniae infection causes mitochondrial damage and dysfunction in bovine mammary epithelial cells. *Vet. Res.* 2021, 52, 17. [CrossRef]

21. Wenz, J.R.; Barrington, G.M.; Garry, F.B.; Dinsmore, R.P.; Callan, R.J. Use of systemic disease signs to assess disease severity in dairy cows with acute coliform mastitis. *J. Am. Vet. Med. Assoc.* 2001, 218, 567–572. [CrossRef]

22. Gurjar, A.A.; Klaessig, S.; Salmon, S.A.; Yancey, R.J., Jr.; Schukken, Y.H. Evaluation of an alternative dosing regimen of a J-5 mastitis vaccine against intramammary *Escherichia coli* challenge in nonlactating late-gestation dairy cows. *J. Dairy Sci.* 2013, 96, 5053–5063. [CrossRef][PubMed]

23. Bagley, S.T.; Seidler, R.J. Primary Klebsiella identification with MacConkey-inositol-carbenicillin agar. *Appl. Environ. Microbiol.* 1978, 36, 536–538. [CrossRef][PubMed]

24. Williams, P.; Brown, M.R.; Lambert, P.A. Effect of iron deprivation on the production of siderophores and outer membrane proteins in Klebsiella aerogenes. *J. Gen. Microbiol.* 1984, 130, 2357–2365. [CrossRef][PubMed]
25. Williams, P.; Smith, M.A.; Stevenson, P.; Griffiths, E.; Tomas, J.M. Novel aerobactin receptor in Klebsiella pneumoniae. J. Gen. Microbiol. 1989, 135, 3173–3181. [CrossRef] [PubMed]
26. Ebrahimie, E.; Mohammadi-Dehcheshmeh, M.; Laven, R.; Petrovsk, K.R. Rule discovery in milk content towards mastitis diagnosis: Dealing with farm heterogeneity over multiple years through classification based on associations. Animals 2021, 11, 1638. [CrossRef] [PubMed]
27. Tsuka, T.; Ozaki, H.; Saito, D.; Murase, T.; Okamoto, Y.; Azuma, K.; Osaki, T.; Ito, N.; Murahata, Y.; Imagawa, T. Genetic characterization of CTX-M-2-producing Klebsiella pneumoniae and Klebsiella oxytoca associated with bovine mastitis in Japan. Front. Vet. Sci. 2021, 8, 659222. [CrossRef]
28. Munoz, M.A.; Welcome, F.L.; Schukken, Y.H.; Zadoks, R.N. Molecular epidemiology of two Klebsiella pneumoniae mastitis outbreaks on a dairy farm in New York State. J. Clin. Microbiol. 2007, 45, 3964–3971. [CrossRef]
29. Smith, K.L.; Todhunter, D.A.; Schoenberger, P.S. Environmental mastitis: Cause, prevalence, prevention. J. Dairy Sci. 1985, 68, 1531–1553. [CrossRef]
30. Compain, F.; Babosan, A.; Brisse, S.; Genel, N.; Audo, J.; Aïlloud, F.; Kassis-Chikhani, N.; Arlet, G.; Decrè, D. Multiplex PCR for detection of seven virulence factors and K1/K2 capsular serotypes of Klebsiella pneumoniae. J. Clin. Microbiol. 2014, 52, 4377–4380. [CrossRef]
31. Döpfer, D.; Almeida, R.A.; Lam, T.J.G.M.; Nederbragt, H.; Oliver, S.P.; Gaastra, W. Adhesion and invasion of Escherichia coli from single and recurrent clinical cases of bovine mastitis in vitro. Vet. Microbiol. 2000, 74, 331–343. [CrossRef]
32. Fairbrother, J.H.; Dufour, S.; Fairbrother, J.M.; Francoz, D.; Nadeau, E.; Messier, S. Characterization of persistent and transient Escherichia coli isolates recovered from clinical mastitis episodes in dairy cows. Vet. Microbiol. 2015, 176, 126–133. [CrossRef] [PubMed]
33. Wenz, J.R.; Barrington, G.M.; Garry, F.B.; Ellis, R.P.; Magnuson, R.J. Escherichia coli isolates’ serotypes, genotypes, and virulence genes and clinical coliform mastitis severity. J. Dairy Sci. 2006, 89, 3408–3412. [CrossRef]