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Genotypic susceptibility, phylotyping and CH clonotyping of porcine *E. coli* isolates

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1. Introduction and hypothesis

Escherichia coli (*E. coli*) was identified 1885 by Theodor Escherich. From the time it was discovered until now, it has become one of the most studied bacterial species. It is easy to grow and is easily manipulated in a laboratory setting. *E. coli* is part of the normal physiological and beneficial flora of the intestine; however, some strains where found to be highly pathogenic and shown to cause disease both in humans and in animals (CLEMENTS et al., 2012).

The current system of food production cannot provide proper safety measures to ensure that meat is not contaminated with strains of *E. coli*, benign as well as pathogenic. Even when safety measures are in place in a facility with high hygienic standards, it is not a guarantee that the meat will not be contaminated. Thus, it could be a potential hazard to humans. Since antimicrobial resistance is on the rise in humans and animals alike, the risk of having meat tainted with bacteria, with any kind of antimicrobial resistance, can increase the hardship of treating *E. coli* outbreaks (RAMADAN et al., 2020).

E. coli can cause three major symptoms in humans: diarrhea, urinary tract infections, and sepsis or meningitis. In farm animals, it is mostly associated with diarrhea especially in pig production. It can cause massive problems due to high mortality rates in piglets. (KAPER et al., 2004; DUBREUIL, 2021; OBALA et al., 2021).

Many studies have found that almost all of current human pathogens are zoonotic or originated in animals before adapting to humans (LLOYD-SMITH et al., 2009; OTTE a. PICA-CIAMARRA, 2021; NAPOLITANO FERREIRA et al., 2021). Not all infections can be classified as being a zoonotic disease. The transmission of human-associated *E. coli* clones from an animal that is carrying those to a human individual cannot be considered a zoonosis, it is considered zooantrophogenic transmission (FONG, 2017).

The aim of the present study was to characterize a total of 102 porcine *E. coli* isolates based on phenotypical resistance genes and sort them into their specific phylogenetic group with the help of quadruplex-PCR and to genotype them with CH-clonotyping. The isolates were collected during routine microbiological examinations at the Institute of Microbiology at the University of Veterinary Medicine Vienna and obtained from isolates from a third party. They were received from third parties and originated from pigs.

The hypothesis of this study was that the isolates originating from pigs do not belong to human-associated clonal complexes.

1.1 Background

1.1.1 *Escherichia coli (E. coli)*

1.1.1.1 Taxonomy

E. coli is a bacterium that belongs to the Phylum *Proteobacteria* where it is in the Class *Gammaproteobacteria* under the Order of *Enterobacterales* and in the Family *Enterobacteriaceae.* Its Genus is *Escherichia,* it is named after Theodor Escherich (Bergey's manual of determinative bacteriology, 2000).

E. coli, when it was first identified, was originally called *Bacterium coli commune* in 1885 by a pediatrician from Austria who published his findings at the medical faculty in Munich, Theodor Escherich. Later it was renamed after Theodor Escherich, who was the first one to isolate this genus (KRIEG et al., 2007).

1.1.1.2 Ecology and habitat

E. coli is a gram-negative, non-sporulating facultative anaerobe that can be found in the intestines and feces of mammals as well as birds, reptiles and amphibia (TENAILLON et al., 2010a; GRÜNZWEIL et al., 2021; ALBER et al., 2021; DEC et al., 2022; CODJO et al., 2022). Since it is facultative, it uses oxygen when it is available, but it also can grow in its absence using fermentation or anaerobic respiration. In the healthy mammal body *E. coli* normally resides in the intestinal tract and forms a symbiosis with gut bacteria. It can benefit the host by producing Vitamin K2 (BENTLEY a. MEGANATHAN, 1982). Only some combinations of virulence factors have become specific pathotypes and can cause diseases in a healthy individual. Three general clinical signs can result from infection with one of these pathotypes: enteric/diarrheal infections, urinary tract infections (UTIs) and sepsis/meningitis. There are six well described intestinal pathogens of *E. coli*: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (KAPER et al., 2004).

1.1.1.3 Morphology

The Cell morphology of this bacteria is that it has straight, cylindrical, Gram-negative rods with round ends. The overall size is 1.1-1.5 µm in diameter and 2.0-6.0 µm in length. They can occur in pairs or solitary. (KRIEG et al., 2007). At best it could be compared to a cylindrical soap bubble. The exoskeleton is made out of three layers: the cytoplasmic membrane, the peptidoglycan or murein layer, and the outer membrane (NANNINGA, 1998). Some *E. coli*

have Flagella that make them motile. Usually they have 5-10 flagella per cell, that are situated in a random pattern around the surface of the cell. These flagella are around 10 nm in diameter and can be up to 20 µm long. The composition of them is a single protein called flagellin. But, not only do they have flagella, most of the strains of *E. coli* also have fimbriae. These are proteins that are situated on the bacterial surface, and they can reach far into the surrounding medium. More than 30 different fimbriae are described in *E. coli*. Most of the time one bacterium expresses more than one of these proteins simultaneously.

Colonial and cultural characteristics depend on the polymerization of the O antigen polysaccharide. They can be described as smooth (S) or rough (R). S forms grow on an agar as convex, glistening, moist, grey colonies with a defined edge while R forms grow as flat, dry, dull, wrinkled colonies with a blurred edge. While S strains have developed polysaccharide side chains, R forms have lost their chains due to mutation. Also, intermediate forms occur (KRIEG et al., 2007).

1.1.1.4 Metabolism and metabolic pathways

E. coli is known to use glucose and other carbohydrates. Using the process of fermentation, it produces lactic, acetic, and formic acids. (KRIEG et al., 2007).

1.1.1.5 In vitro cultivation

This bacteria can be used, grown, and cultured easily in a laboratory setting and has been the topic of investigation for over 60 years (RAUDASKOSKI a. KOTHE, 2010). Most strains grow in a temperature range from 21 °C to 37 °C. There are, however, also strains that can endure temperatures as low as 7.5-7.8 °C. Since *E. coli* is neutrophilic it will grow within a *pH* range of 5.0-9.0 (KRIEG et al., 2007). *E. coli* is the most widely studied organism, and particularly important in the field of microbiology because it has a fast rate of reproduction. Under optimal conditions a generation can grow within just 20 minutes.

1.1.1.6 Methods of typing of *E. coli*

The most common approaches are Multilocus Enzyme Electrophoresis (MLEE) assay, or membrane filtration. (HIGGINS et al., 2007)

Also, Repetitive element-based PCR (REP- PCR), Enterobacterial Repetitive Intergenic Consensus – PCR (ERIC-PCR) and so called BOX- PCR, that is used for identifying flanking regions (combinations of boxA, boxB, and/or boxC) can be both summarized under the abbreviation rep-PCR (MARTIN et al., 1992; BORBA et al., 2020).

In addition, Pulse-field gel electrophoresis (PFGE) can be used to sequence the regions of the genes of interest. The protocol that is closest to a universal approach is the identification of *E. coli* phylogroups through a multiplex PCR approach so that it is possible to sort them into certain groups as later described in 1.1.1.6.7. (CLERMONT et al., 2011). Multilocus Sequence Typing (MLST), Multi-Locus Sequence Analysis (MLSA) and PFGE can be used to determine the clonal relationship between the strains (SHAFIQ et al., 2021).

1.1.1.6.1 Multilocus enzyme electrophoresis (MLEE)

The bacteria are grown overnight. Whether they are grown in nutrient broth or agar plates is not important since electrophoretic mobility is not affected. Cells are harvested and lysed. A device for horizontal electrophoresis is used, and a gel is prepared. Up to 20 lysates can be electrophoresed on one gel. Following electrophoresis slices are cut from the gel and incubated in specific enzyme staining solutions. For each bacterium, the perfect staining must be used. After staining, the gel is analyzed using the comparison of the mobility of the different enzymes (SELANDER et al., 1986).

1.1.1.6.2 Ribotyping

This method requires a pure DNA preparation as described in 2.2. After the DNA is harvested it is digested with restriction enzymes to generate fragments, those are separated by size via electrophoresis. They are then transferred on a nylon membrane and hybridized with a radioactive probe. The fragments that react with the probe are displayed by autoradiography (BINGEN et al., 1994).

1.1.1.6.3 Phylotyping

Using phylotyping, *E. coli* can be sorted in eight different phylo-groups (A, B1, B2, C, D, E, F, clade I). Sorting them into those phylo-groups was performed with a Quadruplex PCR, this allows sorting them either to a definitive group or, using another PCR, to determine to which group the isolate belongs to. This is very important to determine if the type of *E. coli* is pathogenic, and if it is, how pathogenic it is (CLERMONT et al., 2013).

1.1.1.6.4 Membrane filtration

This approach uses control saline solutions and cell dilutions that are passed through gridded cellulose nitrate membrane filters. To identify the *E. coli,* the membranes are transferred to Agar which was stained using 4-methylumbelliferyl-β-d-glucuronide. Under UV the colonies containing *E. coli* fluoresce blue (FOGARTY et al., 2003).

1.1.1.6.5 Repetitive element-based PCR (REP-PCR)

This PCR is widely used due to its speed and cost-effectiveness (HUSSAIN et al., 2021). It is used to fingerprint bacterial genomes by examining a strain-specific pattern that is obtained through PCR amplification of repetitive DNA elements present within the bacterial genome. The fingerprint of each bacteria has a different kind of repetitive element, so this can be used to ID the strain of bacteria (RAMPADARATH et al., 2015).

1.1.1.6.6 Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR)

ERIC sequences are intergenic repetitive units. The goal is to find ERIC sequences because those are distributed differently throughout bacterial species. These sequencies can be used to identify the bacteria. (VERSALOVIC et al., 1991; SUBRAMANIAN et al., 1992; WILSON a. SHARP, 2006).

1.1.1.6.7 Pulse-field gel electrophoresis (PFGE)

This method is used for separation of DNA fragments by alternating an electric field in more than one direction through a solid matrix. Before using this method, the DNA must be prepared using a rare-cutting restriction endonuclease. The visualization and interpretation of the banding pattern can be done on a conventional agarose gel (KAUFMANN, 1998).

1.1.1.6.8 Phylotyping with the Quadruplex PCR

Assigning isolates to a certain phylo-group is very important and due to this significance, a simple method of sorting is needed. A PCR method was developed that could detect *chuA*, *yjaA* and *TspE4.C2*. Then they could be sorted into A, B1, B2 or D but it was detected that although most (80-85 %) were assigned to the right group some of the isolates especially when showing a particular triplex PCR genotype were assigned to the wrong group. So, the revisited Clermont-Method was developed. It uses *arpA* as an internal control, and it distinguishes the strains that belong to group F. This method allows the sorting of strains in the respective group with two

steps. First a quadruplex PCR is performed and then either the strain can be sorted into a group or has to go through another PCR so it can be differentiated between the groups when it is not clear (CLERMONT et al., 2013).

1.1.1.6.9 Multilocus Sequence Typing (MLST)

MLST compares 450-500 base pair fragments of 5-7 housekeeping genes and provides information on the spreading of the nucleotide divergence across the chromosomes of sampled populations (RONG a. HUANG, 2014). There are various protocols to perform MLSTin regard to *E. coli*. The most recent protocol uses internal fragments of the house-keeping genes. These are *adk* (adenylate kinase), *fumC* (fumarate hydratase), *gyrB* (DNA gyrase), *icd* (isocitrate/isopropylmalate dehydrogenase), *mdh* (malate dehydrogenase), *purA* (adenylosuccinate dehydrogenase), and *recA* (ATP/GTP binding motif) (WIRTH et al., 2006a). House-keeping genes are genes that are expressed whether the cell is pathogenic or nonpathogenic (HOUNKPE et al., 2021).There is not only one simple MLST scheme, for example, the Core genome MLST (cgMLST) scheme is considered a tool for detecting a fixed set of core genomes that are conserved within the genome-wide genes. This method is usually used species specific (MAIDEN et al., 1998; MELLMANN et al., 2011).

In the last decade a new Method was developed to simplify genomic analyses. The Whole genome multilocus sequence typing (wgMLST) transforms millions of base pair sequences into data for each gene and makes it easier to compare data between different bacteria or strains (KINGRY et al., 2016).

1.1.1.6.10 Multi-Locus Sequence Analysis (MLSA)

This method was developed from the application of the MLST for reconstruction of evolutionary relationships between the prokaryotes. When evolution takes place, sequences may differ by even one single nucleotide. Thus, making MLSA the perfect tool to see the link and detect the genetic changes between species. The relatedness between the strains in the isolates is obtained by comparing the sequences or allelic profiles (RONG a. HUANG, 2014).

1.1.1.7 Serotypes of *E. coli*

E. coli can be sorted into different groups of Serotypes according to their multiple differences in the antigen structure. This is one of the most useful ways to subdivide the species on a global basis. (KRIEG et al., 2007). They can be sorted into 4 groups:

- **H**-Group: Flagellar H antigens for the flagella, derived from "bacteria growing with a breath", as they actively move around on an agar plate to create a matt ripple pattern.
- **O**-group: Somatic O-antigens, derived from "without a hint" for the lipopolysaccharides that are on the surface of the cell wall.
- **K**-group: K antigens for the capsule, which are composed of polysaccharides.
- **F**-group: fimbrial F antigens for the fimbriae

K and F grousp are rarely used for diagnostic purposes (HAHN et al., 2009).

Infections with Enterohemorrhagic *E. coli* (EHEC) are predominantly caused by *E. coli* serotype O157:H7, but there are multiple serotypes emerging that produce Shiga toxin.

1.1.1.8 Virulence factors of porcine-associated *E. coli*

One of the most important causes of diarrhea in young pigs is ETEC (Enterotoxic *E. coli*), the illness usually occurs in the first week of life or in 3-6-week-old piglets. These symptoms are associated with *E. coli* serotypes, which produce a combination of LT and/or ST enterotoxins and fimbrial colonization factors. Combination of LTI and STI/STII are found in humans as well but only LTII is reported solely in pigs. On other isolates the EAST1 toxin was found in postweaning diarrhea of pigs. Additionally, it was found that pigs who were infected with ETEC also had *E. coli* strains that had one of the pilus-adherence factors: *f*4, *f*5, *f*6, *f*18, or *f*41. Of these factors, *f*4 appears to be the most important one when it comes to adherence of toxigenic *E. coli* to intestinal epithelial cells. The pigs experience, when infected with the ETEC disease, watery diarrhea, because it adheres to the microvilli of the small intestine and produces enterotoxin that acts locally on the enterocytes. This is the cause of hypersecretion and reduced absorption of water. This could end in death for the piglets. To get a hold on this very severe disease, efforts to make autogenous vaccines were made with good results. Antimicrobial resistant strains of *E. coli* were found as early as the early 1960s. Similar reports were collected all over the world that led to the conclusion that most commonly they were resistant to streptomycin, tetracycline, and sulfonamides. There is also evidence that nonantimicrobial selection pressures may maintain antimicrobial resistance which have to be further investigated (AARESTRUP, 2006).In recent years more studies were conducted to look into the virulence

of *E. coli* (HAQ et al., 2020; RUETER a. BIELASZEWSKA, 2020; BERNREITER-HOFER et al., 2021; ANGULO-ZAMUDIO et al., 2021; ZHUGE et al., 2021).

A complete table of virulence-associated genes can be accessed over the INTER-ARREY website (Available online: https://www.inter-array.com/porcineEcoli/VirulenceGenesformanuscript_supplementary_material.xlsx).

2. Material and Methods

2.2 Typing Methods

102 isolates (listed below in Table 11) were obtained during routine bacteriological diagnostics of clinical affected pigs at the Institute of Microbiology of the University of Veterinary Medicine Vienna, Austria and from BS-Immun GmbH Vienna, Austria. They were then provided to be a factor in this study. A one loop of biomass was taken for DNA extraction. For DNA extraction, a GenElute™ Mammalian Genomic DNA Miniprep Kit (Merck Life Science S.r.l. Milan, Italy) was used. The cells were placed in a collection tube where it was resuspended with 200 µL of Resuspension Solution (Sigma - P3980), 40 µL of Proteinase K (Sigma - P2308) was added to lyse the cells, and 30 µL RNase A (Sigma - R6148) was added and incubated 1 min at room temperature. To mix it thoroughly it was vortexed. The tube was then incubated for 15 min at 55 °C with continuous vortexing. Then 200 µl of that solution was added to a new tube and incubated for 1 hour at 55 °C. That was followed by 10 min at 70 ° C incubation to inactivate Proteinase K. Then 500 µl of that solution was transferred into a tube with a GenElute™ Filter; this tube was left to cool down to room temperature. 30 µL RNase A was added to the solution and vortexed, the solutions were left for another 2 min to interact with one another. After this 200 µL 96–100 % ethanol was added to the tube and vortexed, now the whole solution was added to another tube, also prepped with the filter, and put into a centrifuge at 14000 rpm for 2 min. Then the filter was transferred to another tube and 500 µl of Wash Buffer (Sigma-B6553) was added to it and was centrifuged at 14000 rpm for 1 min. The wash process was repeated one time, but the collection tube was centrifuged at 14000 rpm for 3 min. After that the tube was again centrifuged at 12500 rpm for 1 min. The filter was again added to a new tube and 200 µl of Elution Solution (Sigma-B6803) was added; this solution was centrifuged at 12500 rpm for 1 min. Then the spin column was discarded and the DNA residing in the collection tube was frozen at -20 °C. Since this is pure DNA and this much DNA is not needed to perform PCR, the solution was diluted 10 : 1.

2.2.1 Phylotyping of *E. coli*

In which phylo-group the *E. coli* belongs is first determined with a quadruplex PCR, where *chuA.1b*, *chuA.2*, *yjaA.1b*, *yjaA2b*, *TspE4C3.1b*, *TspE4C2.2b*, *Acek.f*, and *ArpAgpE.r* primers where used. Then an electrophoresis is performed, and the *E. coli* can sometimes be immediately assigned to a certain group. If this is not the case, it needs to be further determined by another PCR using the phylotype specific primer or clade primers (Table 1). If the outcome is nonspecific then the isolate should be characterized using the MLST Method. (CLERMONT et al., 2013).

Table 1 Steps of the Quadruplex PCR and determining which group the E. coli belongs to. According to. (CLERMONT et al., 2013)

Quadruplex genotype					
arpA (400 bp)	*chuA* (288bp)	*yjaA* (211bp)	*TspE4.C2* (152bp)	**Phylo-group**	**Next step**
+	-	-	-	A	
+	-	-	+	B1	
-	+	-	-	F	
-	+	+	-	B2	
-	+	+	+	B2	
-	+	-	+	B2	Could be confirmed by testing *ibeA* gene
+	-	+	-	A or C	Screen using C-specific primers. If C+ then C, else A
+	+	-	-	D or E	Screen using E-specific primers. If E+ then E, else D
+	+	-	+	D or E	Screen using E-specific primers. If E+ then E, else D
+	+	+	-	E or clade I	Screen using E-specific primers. If E- then clade I, confirm using cryptic clade primers
-	-	+	-	Clade I or II	Confirm using cryptic clade primers
-	(467)	-	-	Clade III, IV or V	Confirm using cryptic clade primers
-	-	-	+	Unknown	Perform MLST
-	-	+	+	Unknown	Perform MLST
+	-	+	+	Unknown	Perform MLST
+	+	+	+	Unknown	Perform MLST
-	-	-	-	Unknown	Confirm *E. coli* identification using *uidA* or *gadA/B*, if positive screen using cryptic clade primers and /or perform MLST

Table 2 Primers used for Multiplex PCR

PCR reaction	**Primer ID**	**Target**	**Primer sequences**	**PCR product (bp)**	**Volumina (µl)**
Quadruplex	chuA.1b	*chuA*	5`- ATGGTACCGGACGAACCAAC-3´	288	0,3
	chuA.2		5′-TGCCGCCAGTACCAAAGACA-3′		0,3
	yjaA.1b	*yjaA*	5′-CAAACGTGAAGTGTCAGGAG-3′	211	0,3
	yjaA.2b		5′-AATGCGTTCCTCAACCTGTG-3′		0,3
	TspE4C2.1b	*TspE4.C2*	5′-CACTATTCGTAAGGTCATCC-3′	152	0,3
	TspE4C2.2b		5′-AGTTTATCGCTGCGGGTCGC-3′		0,3
	AceK.f	*arpA*	5′-AACGCTATTCGCCAGCTTGC-3′	400	0,6
	ArpA1.r		5′-TCTCCCCATACCGTACGCTA-3′		0,6
Group E	ArpAgpE.f	*arpA*	5′-GATTCCATCTTGTCAAAATATGCC-3′	301	0,3
	ArpAgpE.r		5′-GAAAAGAAAAAGAATTCCCAAGAG-3′		0,3
Group C	trpAgpC.1	*trpA*	5′-AGTTTTATGCCCAGTGCGAG-3′	219	0,3
	trpAgpC.2		5′-TCTGCGCCGGTCACGCCC-3′		0,3
Internal control	trpBA.f	*trpA*	5′-CGGCGATAAAGACATCTTCAC-3′	489	0,3
	trpBA.r		5′-GCAACGCGGCCTGGCGGAAG-3′		0,3

OneTaq® Quick-Load® DNA Polymerase (OneTaq) (New England BioLabs®, USA) was used to perform PCR amplification. All primers were purchased from Invitrogen (Thermo Scientific, Austria) and are listed in Table 2. Primers were used in a 20 pmol pm/µl concentration except for *trpBA.f* and *trpBA.r*, where it was 12 pmol, and for *AceK.f* and *ArpA1.r*, where it was 40 pmol. The master mix was made from distilled water, OneTaq Polymerase, and a forward and reverse primer as described below. The DNA was then added to the mix before putting it into the PCR machine. A Mastercycler® nexus (Eppendorf, Germany) was used for PCR amplification.

2.2.1.1 Multiplex PCR by the Clermont method

All the samples were used to determine in which phylogroup they can be sorted using the primer as listed above in Table 2. The reaction mixture for the amplification using PCR is described in Table 3.

Table 3 Reaction mixture for the Multiplex PCR

Component	**Volume (µl)**
Distilled water	15
OneTaq® Quick-Load® DNA Polymerase	3.5
primers as seen in table 2	
DNA	1

PCR amplification was performed at 94 °C for 4 min, followed by 30 cycles at 94 °C for 5 seconds, 59 °C for 20 seconds, and 72 °C for 2 min. Amplification was finished by a final extension at 72 °C for 5 min.

2.2.2 Two loci multilocus sequence typing (MLST)

To determine the clonotype of the *E. coli* strain in the isolates, a Multilocus sequence typing (MLST) was performed. MLST is currently the preferred method for characterizing the relations between the different bacterial species (WEISSMAN et al., 2012).

2.2.2.1 Mastermix for the two loci MLST PCR

The same material was used as described above (0) but primers were used in a 10 pm/µl concentration. The primers that were used are listed in Table 4

Table 4 Primer used in the Multilocus sequence typing PCR.

Primer ID	Target	Primer sequences	PCR product (bp)	Reference
fumC/F	*fumC*	TCACAGGTCGCCAGC GCTTC	806	(WIRTH et al., 2006b)
fumC/R		TCCCGGCAGATAAGCT GTGG	806	(WIRTH et al., 2006b)
fimH/F	*fimH*	CACTCAGGGAACCATT CAGGCA	975	(WEISSMAN et al., 2012)
fimH/R		CTTATTGATAAACAAA AGTCAC	975	(WEISSMAN et al., 2012)

2.2.2.1.1 PCR amplification of the *fumC* gene

With the MLST, we determined the existence of the house keeping gene *fumC*. Fumarase is widely distributed in organisms because of its important role during the cell metabolism. It is an essential component in the DNA damage response as it protects cells from double-stranded breaks (SILAS et al., 2021).

All the samples were used to determine the existence of the *fumC* gene using the fumC/F and fumC/R. The reaction mixture for the amplification using PCR is described in Table 5.

Table 5 Reaction mixture for fumC or fimH gene amplification.

Component	Volume (µl)
Distilled water	11.25
OneTaq® Quick-Load® DNA Polymerase	15
fumC/F or fimH/F	1.25
fumC/R or fimH/R	1.25
DNA	1.25

PCR amplification was performed at 95 °C for 2 min, followed by 30 cycles at 95 °C for 1 min, 54 °C for 1 min, and 72 °C for 2 min. Amplification was finished by a final extension at 72 °C for 5 min.

2.2.2.1.2 PCR amplification of the *fimH* gene

A two loci MLST was used to determine the presence of the *fimH* gene in all *E. coli* samples using the primer fimH/F and fimH/R. The reaction mixture for the amplification using PCR is described in Table 5.**Fehler! Verweisquelle konnte nicht gefunden werden.**

PCR amplification was performed at 95 °C for 2 min, followed by 30 cycles at 95 °C for 1 min, 57 °C for 1 min, and 72 °C for 2 min. Amplification was finished by a final extension at 72 °C for 5 min.

2.2.2.1.3 Determining the clonal relations of the *E. coli* by CH-clonotyping

After the PCR was performed on the *fumC* and *fimH* genes the amplicons were loaded on an electrophoresis gel as described below (2.2.3). The products that had a positive match with the length of the bp were sent to LGC Biosearch Technologies (Ostendstraße 25, 12459 Berlin, Germany as described below (2.2.4). After being provided with the exact sequence of the amplicon it was uploaded into the CHTyper (CGE Server, 04.06.2021) and it was confirmed using the PubMLST Database (https://pubmlst.org/bigsdb?db=pubmlst_escherichia_seqdef&page=sequenceQuery, last accessed on 24.08.2022). With the combination of the two-locus sequence-based typing scheme of the *fumC* and the *fimH* gene the clonal relatedness can be determined (WEISSMAN et al., 2012).

2.2.3 Electrophoresis of PCR products

2.2.3.1 Preparing the Agarose gel

PCR amplicons were separated using a 2 % (w/v) agarose gel. It was prepared by dissolving 6 g. agarose (LabQ Standard Agarose LE) in 300 ml 1 x TBE buffer. Then the suspension was heated until all the particles have been dissolved. The 1 x TBE buffer was obtained by diluting the 10 x TBE buffer. Contents of the 10 x TBE buffer are as listed below in Table 6. All the ingredients were ordered from Carl Roth GmbH + Co. KG, Germany.

Table 6 Content of the 10x TBE buffer

Ingredient	**Volume**
Tris Pufferan ® ≥99,9 %, p.a.	108 g
Boric acid	55 g
Ethylenediaminetetraacetic acid (EDTA)	9.3 g
Distilled water	1000 ml

2.2.3.2 Loading the gel

For the separation, a gel electrophoresis was performed. It was performed in an electrophoresis chamber. 7 µl of the target PCR amplification product and 4 µl of a molecular marker (NEB® 2-Log DNA Ladder, New England BioLabs®, USA) were loaded onto the electrophoresis gel.

2.2.3.3 Staining of the gel

The Gel was stained with a 2.5 mg/l ethidium bromide solution for approximately 20 min and then washed in distilled water.

2.2.3.4 Visualization of the DNA band pattern

The DNA band pattern was visualized using a ChemiDocTM MP Imaging System (Bio-Rad Laboratories, Germany).

2.2.4 DNA Sequencing

After identifying the existence of *fumC* or *fimH* genes in the PCR products, the amplicons were sent to LGC genomics for sequencing.

2.2.4.1 PCR clean-up Process

Two PCR clean-up Processes were used to remove the remaining primers from the PCR product before sending 25 pmol to LGC genomics.

2.2.4.1.1 PCR clean-up Exo one

The first protocol that was used was the Enzymatic PCR clean up using Exonuclease I (Exo I, NEB #M0293) and Shrimp Alkaline Phosphatase (rSAP, NEB #M0371) (BIOLABS, 04.06.2021a). The exact content of the reaction mixture is listed in Table 7 .

Table 7 Content of the reaction mixture for the PCR clean-up

Component	**Volume (µl)**
Exo I	1
rSAP	2
PCR product	10

PCR amplification was performed at 37 °C for 15 min, followed by 1 cycle at 80 °C for 15 min.

The content of the transport tubes was as listed in Table 8 .

Table 8 Content of the transport tubes

Component	**Volume (µl)**
PCR clean-up product	7.5
Distilled water	6
Forward Primer of the target gene	2.5

2.2.4.1.2 PCR clean-up protocol two

The second PCR clean-up protocol used was the Enzymatic PCR clean up using Exonuclease Calf Intestinal Phosphatase A and B (Exo-CipA and Exo-CipB) (New England Biolabs GmbH Brüningstr. 50; Geb. B852 D-65926 Frankfurt am Main, 04.06.2021b). The exact content of the reaction mixture is listed in Table 9.

Table 9 Content of the reaction mixture for the PCR clean-up

Component	**Volume (µl)**
Exo-CipA	1
Exo-CipB	1
PCR product	10

PCR amplification was performed at 37 °C for 4 min, followed by one cycle at 80 °C for 1 min. The content of the transport tubes is listed in Table 10.

Table 10 Content of the transport tubes

Component	**Volume (µl)**
PCR clean-up product	7.5
Distilled water	5.5
Forward Primer of the target gene	2.5

3 Results

3.2 Phylotyping of *E. coli*

Out of 102 samples of *E. coli* most of them were found to belong to group A (n = 53). The second most common group was B1 with 26 of the samples belonging to this group. Nine were found to belong to group C, five to group D and two were found to belong to group F. One isolate each could be assigned to the groups E, G, and clade 1 as seen in Table 11.

3.3 Genotyping of *E. coli*

The *fumC* and *fimH* genotyping divided the *E. coli* isolates into 51 distinct CH clonotypes and revealed clonal relatedness of 12 *E. coli* isolates (CH27-0), 9 isolates (CH11-54) and 8 isolates (CH11-23). *E. coli* predicted CH-Clonotype CH40-24 was clearly determined in isolates 24-8 and 99-74. Relatedness of isolates is visualized in Figure 1.

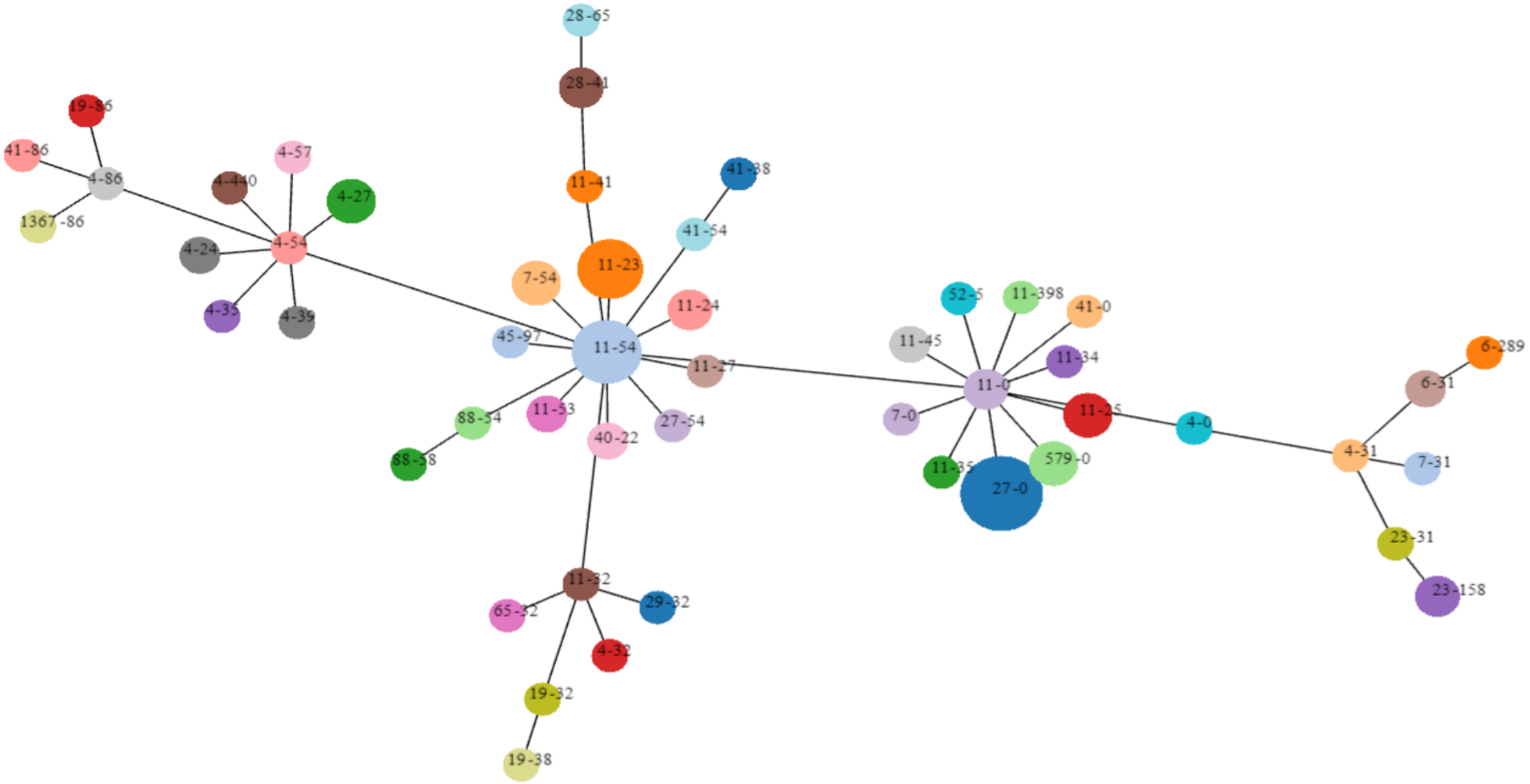

Figure 1 goeBURST diagram for the CH clonotyping data set of E. coli isolates. An eBURST diagram was calculated using PHYLOViZ with the goeBURST algorithm. E. coli isolates were grouped according to their CH profiles (BERNREITER-HOFER et al., 2021)

Table 11 Distribution of Phylogroup, CH-clonotyping of each E. coli isolates used in this study.

Sample number	**Phylogroup**	**CH-clonotyping**
46_30/48_32	A	11-0
66_41	B1	11-0
3651/4245/108_83/21_5/47_31/75_50/79_54	A	11-23
40_24	B1	11-23
104_79	B1	11-24
76_51/80_55	A	11-24
49_33/84_59	B1	11-25
89_64/92_67	A	11-25
38_22	B1	11-27
37_21	A	11-32
96_71	B1	11-34
88_63	A	11-35
93_68	B1	11-398
27_11	B1	11-41
73_48/74_49	A	11-45
107_82	A	11-53
23_7	G	11-53
20_4/43_27/72_47/81_56/3835_4	A	11-54
36_20	D	11-54
4347_1/61_36	B1	11-54
106_81	clade 1	1367-86
566_1	D	19-32
62_37	A	19-38
2945_3	C	19-86
40541_1	C	23-158
51_15	B1	23-158
85_60	A	23-158
3730	B1	23-31

Table 12continued

Sample number	Phylogroup	Ch-clonotyping
1450/101_76/448_2/45_29/68_43/69_44/90_65/91_66	A	27-0
103_78	C	27-0
109_84	B1	27-0
64_39	B2	27-0
77_52	E	27-0
98_73	A	27-54
566_2/566_3	D	28-41
60_35	A	28-41
19_3	C	28-65
22_6	A	29-32
17_1	A	4-0
25_9	A	40-22
3835_2	B1	40-22
87_62	C	41-0
70_45	B1	41-38
67_42	A	41-54
95_70	A	41-86
35_19/40541_2/97_72	A	4-24
82_57/41_25/86_61	B1	4-24
28_12	F	4-31
94_69	B1	4-32
33_17	A	4-35
448_1	A	4-39
3835_3	A	4-440
30_14	A	4-54
42_26	A	4-57
24_8	B2	45-97
50_34	B1	4-86
65_40	A	52-5

Table 12 continued

Sample number	Phylogroup	Ch-clonotyping
4347_2/4347_3	B1	579-0
44_28	C	579-0
71_46	A	579-0
83_58	B2	6-289
105_80	B1	6-31
39_23	C	65-32
34_18	C	7-0
630_2/ 78_53	A	7-31
18_2	D	7-54
32_16	C	7-54
63_38	B1	7-54
99_74	B2	7-54
26_10	B1	88-54
29_13	F	88-58

4 Discussion

In the present study, 102 *E. coli* isolates from porcine individuals were cultivated, and the DNA was extracted to sort them into different phylotypic groups to understand which genotypes they belong to. Sadly, while researching this topic, it was noticed that not a lot of studies were published regarding pigs and *E. coli* with these kinds of phylotypes or clonotypes up to date, therefore it was sometimes hard to compare this data to previous studies.

E. coli is one of the best characterized bacterial models (TENAILLON et al., 2010b). Understanding what geno- and phylotype they belong to help us understand how pathogenic these strains are (JOHNSON a. STELL, 2000). In combination with what group of virotype they belong to. In general, these groups are used to describe the relatedness of certain strains to each other and offer another way to examine biodiversity (NIXON, 2013). It was established that there are four groups: A, B, C and D. While strains of groups A and B show multidrug resistance, group C was shown to cause statistically higher rates of infection (MATHERS et al., 2015a).

There are numerous different methods of sorting them into different phylogroups, but the most convenient and the most beneficial for this study was the Clermont Method since it is one of the only Methods that concentrates all the information needed (CLERMONT et al., 2013).

The results indicate that the most common isolates belong to group A (n = 53) and group B1 (n = 26). In various other studies it was found that this distribution was the most common occurrence regarding *E. coli* (WHITE et al., 2011; NICOLAS-CHANOINE et al., 2014; PETIT et al., 2017; SARACENO et al., 2021; COOKSON et al., 2022). The majority of the *E. coli* isolates that belong to group A and B1 in humans are non-pathogenic whereas in pigs those are the groups that harbor extraintestinal pathogenic *E. coli* (BOK et al., 2020). This indicates that it is important to understand what kind of *E. coli* strain is being worked with and how it could affect the hosts. This study was part of another study published that also investigated the antimicrobial resistance of each sample. The results manifested that out of all the samples, 76 % were resistant to at least one antibiotic. Out of these, the majority of resistances was found against penicillins (61.73 %) and tetracyclines (58.81 %). (BERNREITER-HOFER et al., 2021). This reveals how significant for human and animal medicine it is to have proper testing before giving antibiotics since the result of using broad spectrum antibiotics leads to over half of those strains already being resistant to at least one of

those antibiotics that are commonly used by humans to treat any kind of diseases (ABUSHAHEEN et al., 2020).

It is a prime goal to understand how pathologic these strains are since antimicrobial resistance is growing. The potential of the zoonotic diseases that are resistant to most commonly used antibiotics is a major concern (YANG et al., 2004). Since the animals we are testing are predominantly used for human consumption, it is paramount to understand if there is a correlation between certain genotypes and antimicrobial resistance.

As mentioned above this study is part of a larger one where 35 isolates were whole genome sequenced and 16 different STs were found (BERNREITER-HOFER et al., 2021). The most repeatedly found type was ST10 that is known to cause diarrhea in bovines and wild birds as well as proclaimed to cause more instances in ICUs of multidrug resistance. This sequence type was also found commonly in northern European Pigs that had been diagnosed with ETEC (SHEPARD et al., 2012; CANTÓN et al., 2020; HE et al., 2021).

ETEC causes infections in newborn, suckling, and in post-weaning piglets, and is responsible for diarrhea in farm animals as well as in humans. The enterotoxins that are expressed by ETEC play a central role in the pathogenic process that can reduce growth rate, morbidity, and mortality (DUBREUIL, 2021).

Furthermore, ST100 that was detected in our isolates was discovered to cause diarrhea in postweaning pigs and also have a high rate of antimicrobial resistance and a high diversity of virulence genes (GARCÍA et al., 2020). From 35 isolates 5 showed to be from the type ST354, that indicates to have a high potential of causing zoonotic outbreaks (ZHUGE et al., 2021). One of the STs that was detected was ST131. *E. coli* that belong to ST131 are commonly isolated from human and animal specimens and from environmental origin (https://enterobase.warwick.ac.uk/species/index/ecoli, accessed on 10 August 2022). This clone has been characterized as a pandemic high-risk clone (MATHERS et al., 2015b). ST131 has 13566 entries in the Enterobase Escherichia/Shigella Database (https://enterobase.warwick.ac.uk/species/index/ecoli, accessed on 10 August 2022) and has been-associated with various clinical symptoms such as bacteremia, colibacillosis, cystitis, diarrhea, gingivitis, meningitis, pyelonephritis, respiratory tract infections, septicemia, urinary tract infection (UTI) and wound infections from both humans and animals. Pandemic character of the ST131 is clearly indicated by isolation of this clone from 93 countries.

In addition, the multi-drug resistant character of the ST131 isolate characterized during the present study confirmed this well-known characteristic of this clone (MATHERS et al.,

2015b). This clone is part of the ExPec (PITOUT, 2012). It can adapt more rapidly to certain environments than others thus making it a more dangerous variant (MATHERS et al., 2015b). ST10, ST100, ST345 and ST131 were the ones that were found the most in these samples, but there were also new sequence types obtained: ST12008 (37_21), ST12009 (46_30), ST12010 (98_73) which still need to be properly characterized.

Clonal relatedness was also a subject in this study. Not only it is important to understand which strains were found but also what kind of common ground these strains share. If we look at various isolates and compare them to different studies that were made with that kind of CH-Clonotype regarding their virulence factors, we can look at the outcome and how pathogenic they were to be found. Since comparison is a big part in finding out new strains or finding new diseases that are linked to this CH-Clonotype with those kind of virulence factors. The most common CH-Clonotype within our samples was 27-0 (12 %) followed by 11-23 and 11-54 (both 8 %). 4-24,7-54,11-25, and 579-0 where present (4 %). 11-0,11-24,23-158, and 28-41 were found (3 %) and 4-24,7-31, 11-53,11-45, and 40-22 (each 2 %). An alarming prevalence of virulence factors were found in our isolates. The most predominant gene was *astA* followed by *itcA* (BERNREITER-HOFER et al., 2021).This study correlates with a study that used a 12 year period to determine what kind of CH-clonotypes of *E. coli* were found. One of the clonotypes that was found in both was CH40-22. (MAMANI et al., 2019). Furthermore, a study showing matching CH-Clonotypes with antimicrobial selection can significantly reduce the mismatch compared to conventional therapy. The decrease of a mismatch was ranged between 62 – 78 %. This is very important since antimicrobial resistance is on the rise. When researching ways to improve conventional therapy, one should always think one step further than common knowledge (TCHESNOKOVA et al., 2013).

5. Disclaimer

This Study was part of a larger study (The Pheno- and Genotypic Characterization of Porcine *Escherichia coli* Isolates) and some of the Data mentioned here was kindly provided by the authors of this study.

6. Summary

E. coli is an important pathogen in veterinary medicine that is highly published in the biomedical community, not only across Europe, but the world. In the present study, a total of 102 isolates of *E. coli* were geno- and phenotypically characterized using Multiplex PCR, via the Clermont Method, MLST for determining the existence of *fimH* and *fumC* genes, and to prove the clonal relations of *E. coli* by CHtyping. Out of 102 samples of *E. coli* most of them were found to belong to group A (n = 53). The second most common group was B1 (n=26). Nine were found to belong to group C, five to group D, and two were found to belong to group F. One isolate each could be assigned to the groups E, G, and clade 1. The *fumC* and *fimH* genotyping divided the *E. coli* isolates into distinct CH clonotypes and revealed clonal relatedness of 12 *E. coli* isolates (CH27-0), 9 isolates (CH11-54), and 8 isolates (CH11-23). *E. coli* predicted CH-Clonotype CH40-24 was clearly determined in isolates 24-8 and 99-74. Relatedness of isolates was visualized by a dendrogram. Another study was done in accordance with this one that showed 76 % of analyzed samples were also resistant to at least one antibiotic, most commonly to penicillins (61.73 %) and tetracyclines (58.81 %). In addition, 35 of the samples where whole genome sequenced to fully characterize them. The most important finding was ST131 which is one of the strain with the most potential among the strains regarding antimicrobial resistance and its ableness to adapt to almost everything This study is only one step to fully understanding the different clonotypes and how transmissible they are to humans. Further studies can help to understand how to mitigate antimicrobial resistance (AMR) and to investigate new possibilities for alternative therapies.

7. Zusammenfassung

E. coli ist ein wichtiges pathogen in der Veterinärmedizin über das viel in der Biomedizinischen Gemeinschaft publiziert wird, nicht nur in Europa, sondern auch auf der ganzen Welt. In dieser Studie wurden 102 Isolate von *E. coli* geno – und phenotypisiert mithilfe der Multiplex PCR Methode von Clermont, MLST für die Bestimmung der Existenz von *fimH* und *fumC* Genen und der Überprüfung der Verwandtschaftsverhältnisse bei den einzelnen Stämmen zueinander mithilfe des CHtypings. Von 102 Proben gehörten die meisten zur Gruppe A (n = 53). Die zweit größte Gruppe war B1 mit 26 Proben. Neun gehörten zur Gruppe C, fünf zur Gruppe D und bei zwei wurde festgestellt das sie zur Gruppe F gehörten. Jeweils ein Isolat konnte der Gruppe E, G und clade 1 zugeordnet werden. Die *fumC* und *fimH* Genotypisierung ordnete die *E. coli* Isolate in bestimmte CH clonotypen ein und die Verwandtschaft dieser konnte bewiesen werden. 12 der Proben gehörten zur CH27-0, neun zu

CH11-54 und acht zu CH11-23. *E. coli* des CH- Clonotyp CH40-24 wurde in den Isolaten 24-8 and 99-74 festgestellt. Die Verwandtschaftsverhältnisse wurden mittels eines Dendrogramms visualisiert. Diese Studie wurde in Zusammenarbeit einer weiteren getätigt die sich unter anderem mit Antibiotikaresistenz beschäftigte. Die Daten dieser zeigen das 76 % gegen Antibiotika resistent sind, darunter 61,73 % gegen Penicilline und 58,81 % gegen Tetracycline. Zusätzlich wurden 35 der Proben Vollgenom sequenziert, um sie vollständig zu charakterisieren. Der wichtigste Fund dabei war ST131, das derzeit zu den wichtigsten Stämmen dieser Zeit gehört da es nicht nur eine hohe Rate an Antibiotikaresistenz besitzt, sondern auch da es sich sehr anpassungsfähig an fast alle Umstände zeigt. Diese Studie ist nur ein Schritt zum Vollkommenen Verständnis der verschiedenen Clonotypen und wie übertragbar diese auf Menschen sein können, weitere Studien werden gebraucht, um das Verständnis in Bezug zu Antibiotikaresistenz zu erhöhen und Möglichkeiten zu finden, um den Antibiotikaeinsatz zu verringern. Diese Studie ist nur ein Schritt zum vollständigen Verständnis der verschiedenen Clonotypen und ihrer Übertragbarkeit auf den Menschen. Weitere Studien können dazu beitragen, zu verstehen, wie Antibiotikaresistenz entgegengewirkt werden kann, und neue Möglichkeiten für alternative Therapien zu einer konservativen Antibiotika Therapie zu untersuchen.

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9. List of figures and tables

10. List of abbreviation

E. coli ..... *Escherichia coli*

PCR ..... Polymerase chain reaction

UTI ..... Urinary Tract Infection

EPEC ..... Enteropathogenic *E. coli*

EHEC ..... Enterohemorrhagic *E. coli*

ETEC ..... Enterotoxigenic *E. coli*

EAEC ..... Enteroaggregative *E. coli*

DAIC ..... Diffusely adherent *E. coli*

nm ..... nanometer

µm ..... micrometer

pH ..... potential of hydrogen

MLEE ..... Multilocus enzyme electrophoresis

REP – PCR ..... Repetitive Element-Based PCR

ERIC – PCR ..... Enterobacterial Repetitive intergenic Consensus PCR

PFGE ..... Pulse – field Gel Electrophoresis

MLST ..... Multilocus Sequence Typing

MLSA ..... Multilocus Sequence Analysis

DNA ..... Deoxyribonucleic acid

ID ..... Identifier

BPW ..... Buffered Peptone Water

TBE ..... Tris Borat EDTA Buffer

EDTA ..... Ethylenediaminetetraacetic acid rSAP Shrimp Alkaline Phosphatase

Exo – CipA/B ..... Exonuclease Calf Intestinal Phosphatase A/B

11. Appendix

microorganisms

MDPI

Article

The Pheno- and Genotypic Characterization of Porcine *Escherichia coli* Isolates

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Abstract: *Escherichia* (*E.*) *coli* is the main causative pathogen of neonatal and post-weaning diarrhea and edema disease in swine production. There is a significant health concern due to an increasing number of human infections associated with food and/or environmental-borne pathogenic and multidrug-resistant *E. coli* worldwide. Monitoring the presence of pathogenic and antimicrobialresistant *E. coli* isolates is essential for sustainable disease management in livestock and human medicine. A total of 102 *E. coli* isolates of diseased pigs were characterized by antimicrobial and biocide susceptibility testing. Antimicrobial resistance genes, including mobile colistin resistance genes, were analyzed by PCR and DNA sequencing. The quinolone resistance-determining regions of *gyrA* and *parC* in ciprofloxacin-resistant isolates were analyzed. Clonal relatedness was investigated by two-locus sequence typing (CH clonotyping). Phylotyping was performed by the Clermont multiplex PCR method. Virulence determinants were analyzed by customized DNA-based microarray technology developed in this study for fast and economic molecular multiplex typing. Thirty-five isolates were selected for whole-genome sequence-based analysis. Most isolates were resistant to ampicillin and tetracycline. Twenty-one isolates displayed an ESBL phenotype and one isolate an AmpC β-lactamase-producing phenotype. Three isolates had elevated colistin minimal inhibitory concentrations and carried the *mcr-1* gene. Thirty-seven isolates displayed a multi-drug resistance phenotype. The most predominant β-lactamase gene classes were $bla_{\text{TEM-1}}$ (56%) and $bla_{\text{CTX-M-1}}$ (13.71%). Mutations in QRDR were observed in 14 ciprofloxacin-resistant isolates. CH clonotyping divided all isolates into 51 CH clonotypes. The majority of isolates belonged to phylogroup A.

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Sixty-four isolates could be assigned to defined pathotypes wherefrom UPEC was predominant. WGS revealed that the most predominant sequence type was ST100, followed by ST10. ST131 was detected twice in our analysis. This study highlights the importance of monitoring antimicrobial resistance and virulence properties of porcine *E. coli* isolates. This can be achieved by applying reliable, fast, economic and easy to perform technologies such as DNA-based microarray typing. The presence of high-risk pathogenic multi-drug resistant zoonotic clones, as well as those that are resistant to critically important antibiotics for humans, can pose a risk to public health. Improved protocols may be developed in swine farms for preventing infections, as well as the maintenance and distribution of the causative isolates.

Keywords: antimicrobial resistance; pig; *E. coli*; molecular characterization; microarray; colistin; WGS

1. Introduction

Escherichia (*E.*) *coli* is a facultatively anaerobic Gram-negative rod with many facets. The majority of *E. coli* strains inhabit the intestinal tract of humans and warm-blooded animals as commensal bacteria in a mutually beneficial association with its hosts [1–3]. However, some strains of *E. coli* have acquired virulence-associated genes (VAGs), rendering them pathogenic and empowering them to play an important role as pathogens in humans and animals [3]. *E. coli* is a prominent cause for a wide range of bacterial infections in swine but might

also play a role as a bacterial foodborne pathogen. In particular, VAGs enable *E. coli* to cause enteritis, urinary tract infections, peritonitis, meningitis, and septicemia in humans. In swine, *E. coli* is more prominently associated with diarrhea [4]. Depending on their VAGs, their patho-mechanisms and their clinical symptoms, *E. coli* strains are classified into numerous pathotypes. Diarrhea-associated strains include enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), and enteroinvasive *E. coli* (EIEC). Extraintestinal infections are caused by extraintestinal pathogenic *E. coli* strains (EXPEC). EXPEC are mostly innocuous gut commensals that are harmful only if they reach other body sites. They include uropathogenic strains (UPEC), or strains that are involved in septicemia in humans and animals (SEPEC), as well as *E. coli* that are involved in neonatal meningitis of humans (MENEC) [4–6].

E. coli represents a versatile and diverse enterobacterial species with a broad genetic flexibility and adaptability to constantly changing environments [7]. *E. coli* has acquired antimicrobial resistance mechanisms [8]. The genetic adaptation of *E. coli* to antibiotic exposure may select for decreased susceptibility to several antimicrobial agents [9]. Antimicrobial resistance (AMR) is recognized as a global problem in human and veterinary medicine. The high prevalence of multidrug-resistant (MDR) bacteria causes a significant concern in public health [10]. The extended use of critically important antibiotics in livestock also affects the emergence, prevalence, and dissemination of AMR [11]. VAGs and antimicrobial resistance genes are often carried on mobile genetic elements that might enable zoo-anthropogenic transfer. Therefore, monitoring the presence of pathogenic and drug-resistant *E. coli* isolates is essential for sustainable disease management in livestock and human medicine [12].

The testing and screening of virulence genes of porcine *E. coli* by single and/or multiplex PCRs is an economic factor in the frame of routine microbiological diagnostics [13]. There are numerous VAGs, but a limited number of them are usually examined by a combination of single or multiplex PCRs [13]. Accurate and time saving determination of a wide variety of genes can be accomplished using DNA microarray-based assays [14]. In the present study, we developed a microarray-based diagnostic tool combining oligonucleotides designed to detect a customized set of VAGs for use in routine diagnostics.

In Austria, there is a limited body of data describing the genomic epidemiology of

E. coli from swine. Therefore, the objective of the present study was to characterize porcine

E. coli, isolated during routine diagnostics, by a polyphasic approach including pheno- and genotypic susceptibility testing and whole-genome sequencing of selected isolates. For the rapid identification of virulence genes in *E. coli*, customized DNA microarray assay were developed within this study.

2. Materials and Methods

2.1. E. coli Isolates

A total of 102 *E. coli* isolates of suckling and weaning pigs were included in the present study. All isolates were gut-associated and were obtained during routine bacteriological diagnostics at the Institute of Microbiology of the University of Veterinary Medicine Vienna, Austria and from BS-Immun GmbH Vienna, Austria. All isolates originated from clinical samples received from third parties and therefore were not subject to reporting obligations of the Ethics and Animal Welfare Commission of the University of Veterinary Medicine Vienna. Isolates were stored at −80 °C until further examination.

2.2. Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed by agar disk diffusion according to the CLSI [15]. *Escherichia coli* ATCC® 25,922 served as the quality control strain. The following antimicrobials were used: ampicillin (10 µg), piperacillin (10 µg), cefotaxime (30 µg), ceftazidime (30 µg), cefepime (30 µg), aztreonam (30 µg), meropenem (10 µg), imipenem (10 µg), gentamicin (10 µg), amikacin (30 µg), tobramycin (10 µg), ciprofloxacin (5 µg), trimethoprim–sulfamethoxazole (1.25/23.75 µg), tetracycline (30 µg), fosfomycin (200 µg), and chloramphenicol (30 µg) (Becton Dickinson, Heidelberg, Germany). Isolates were further examined for extended-spectrum β-lactamase (ESBL) production by combination disk tests using cefotaxime and ceftazidime with and without clavulanic acid (Becton Dickinson, Heidelberg, Germany) [15]. Furthermore, cefoxitin (30 µg)

(BD, Heidelberg, Germany) was utilized to detect AmpC β-lactamase-producing (AmpC) phenotypes. Minimal inhibitory concentration of isolates mobile colistin resistance (*mcr*) determinants were screened by broth microdilution testing method in accordance with the CLSI document VET01-A4 [16]. Colistin susceptibility testing was interpreted according to the CLSI document MR01 [17]. Escherichia coli ATCC® 25,922 served as quality control strain. *E. coli* isolates displaying the AmpC phenotype were analyzed for mutations in the chromosomal *ampC* promoter/attenuator region as described previously [18]. The following resistance genes were screened via PCRs: *bla*$_{CMY}$, *bla*$_{CTX}$, *bla*$_{OXA-1}$, *bla*$_{OXA-2}$, *bla*$_{SHV}$, *bla*$_{TEM}$, *sul1*, *sul2*, *sul3*, *dfrA1*, *dfrA12*, *dfrA14*, *dfrA17*, *dfrA19*, *strA*, *strB*, *aadA1*, *aadA2*, *aadA4*, *aadA5*, *aadB*, *qepA*, *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6^0)-Ib-cr*, *catA1*, *cfr*, *cmlA1*, *floR*, *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G) as described elsewhere [19,20]. In addition, the genes *bla*$_{CMY}$, *bla*$_{CTX-M}$, *bla*$_{SHV}$, and *bla*$_{TEM}$ were sequenced after PCR amplification. All amplicons in the present study were sequenced at LGC Genomics, Berlin, Germany. Sequences were aligned with BLAST (Basic Local Alignment Search Tool. Available online: https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 29 July 2021) and compared with reference sequences available in GenBank and the National Center for Biotechnology Information (NCBI) database (Beta Lactamase Data Resources. Available online: http://www.ncbi.nlm.nih.gov/pathogens/beta-lactamase-data-resources/, accessed on 29 July 2021). PCR for plasmid-mediated colistin resistance genes, *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5*, was performed according to the protocol of European Union Reference Laboratory for Antimicrobial Resistance [21]. The quinolone resistance-determining regions (QRDR) of *gyrA* and *parC* in ciprofloxacin-resistant isolates were amplified by PCR and sequenced [22].

2.3. Bicocide Susceptibility Testing

Biocide susceptibility testing was performed according to the previously established protocol by Schug et al. [23]. Established minimal inhibitory concentration (MIC) values of investigated biocides on reference strains are shown in Supplementary Materials Table S2. Benzalkonium chloride (Acros Organics, Geel, Belgium, 21541), as a representative of the quaternary ammonium compounds, was tested at concentration ranges 0.000015–0.016%; chlorhexidine (Sigma-Aldrich, Schnelldorf, Germany, 55-56-1), as a representative of cationic compounds, was tested at concentration ranges 0.000015–0.002%; glutardialdehyde (Chempur, Piekary Slaskie, Poland, 424610240), as a representative of aldehydes, was tested at concentration ranges 0.0075–1%; and isopropanol (99.9%, PHPU Eurochem BGD, Tarnow, Poland), as a representative of alcohols, was tested at concentration ranges 1–14%. The method was performed in 96-well polystyrene microtiter plates with U bottom (Sarstedt, Numbrecht, Germany, 82.1582.001). The bacterial inoculum was prepared according to the CLSI standard (Clinical and Laboratory Standards Institute, 2020), using Trypticasein soy broth (BioMaxima, Lublin, Poland, PS 23-500). The final concentration of bacteria inoculated into the wells was 2.5–5 × 10^5 CFU/mL.

2.4. Clonal Relatedness of E. coli and Whole-Genome Sequencing

E. coli DNA was extracted as previously described [24]. Isolates were phylotyped using the quadruplex assignment method [25]. Clonal relatedness of *E. coli* isolates was assessed by two-locus sequence typing, or "CH-clonotyping", using combined data of *fumC* and *fimH* sequences as described by Weissman et al. [26]. Allele and CH clonotype numbers were used for goeBURST analysis using PHYLOViZ [27]. Thirty-five selected *E. coli* isolates were analyzed by whole-genome sequencing (WGS), which was performed by isolating bacterial DNA using the MagAttract HMW DNA Kit (Qiagen, Hilden, Germany). Ready-to-sequence libraries were prepared using Nextera XT DNA Library Preparation

Kit (Illumina, San Diego, United States). Sequencing was performed on the Illumina MiSeq platform [28]. De novo assembly of the 300 bp paired-end reads was conducted using SPAdes 3.9.0 [29]. WGS data analysis was performed with SeqSphere+ software (Ridom, Münster, Germany). To assess the genetic relatedness between the *E. coli* isolates, multi-locus sequence typing (MLST) and core genome multi-locus sequence-based typing (cgMLST) were performed as previously described [30]. To identify acquired resistance genes or chromosomal mutations, Comprehensive Antibiotic Resistance Database [31] as well as ResFinder 4.1 [32,33] were used. Genes associated with biocide resistance were compared with BacMet database (Antibacterial Biocide and Metal Resistance Genes Database. Available online: http://bacmet.biomedicine.gu.se/, accessed on 29 July 2021) [34]. Virulence genes were identified using VirulenceFinder [35,36]. CH types were characterized as mentioned above. Serogenotypes were analyzed by SerotypeFinder [37]. *E. coli* phylotypes were extracted

from WGS by Clermont typing [38]. The presence of plasmids was determined using PlasmidFinder [39]. Probability prediction of the location of a given virulence or antibiotic resistance gene was achieved by applying mlplasmids trained on *E. coli* [40]. Posterior plasmid probability (ppp) scores ≥0.7 at a minimum contig length of 700 bp indicate that a given contig sequence is plasmid-derived. For selected contigs with lower ppp scores, BLAST analyses against the *Enterobacterales* nucleotide collection at NCBI were performed. Plasmid probability was assumed for mlplasmid scores > 0.699 or if BLAST analyses identified *E. coli* plasmids for at least 90% of contig length with >90% identity. The genomes of WGS isolates were deposited under PRJNA728557 in the NCBI BioProject database.

2.5. Microarray-Based Detection of Virulence-Associated Genes

A set of virulence genes was determined for all isolates using a DNA microarray-based technology developed in the present study frame. The technology is based on methods as described previously [41], and custom-made microarrays from INTER-ARRAY (INTERARRAY by fzmb GmbH, Bad Langensalza, Germany) were used according to manufacturer's instructions. The complete list of virulence-associated genes can be found at INTERARRAY website (Virulence Genes for Manuscript. Available online: https://www.interarray.com/porcineEcoli/VirulenceGenesformanuscript_supplementary_material.xlsx, accessed on 29 July 2021). A split network tree was used to visualize similarities between hybridization patterns as described previously [13].

3. Results

3.1. Antimicrobial Susceptibility Testing

All isolates were susceptible to amikacin and carbapenems. Out of the 102 *E. coli* strains, 79.41% were resistant to at least one of the remaining antimicrobial agents tested. Twenty-one isolates displayed an extended-spectrum β-lactamase (ESBL) phenotype, whereas a single isolate displayed an AmpC phenotype. In total, 36.27% of the isolates exhibited an MDR phenotype [10]. The majority of isolates were resistant to ampicillin (61.75%) and/or tetracycline (58.81%). Further resistance rates were found to piperacillin (26.46%), sulfamethoxazole–trimethoprim (23.53%), cefotaxime (13.71%), chloramphenicol (11.75%), ceftazidime (8.81%), cefepime (7.83%), gentamicin (6.85%), fluoroquinolone (5.87%), aztreonam (4.90%), tobramycin (3.91%), and fosfomycin (1.95%). A total of 2.94% of all investigated isolates exhibited elevated colistin MICs of ≥4 µg/mL. All results of antimicrobial susceptibility testing are summarized in Tables 1 and 2.

Table 1. Pheno- and genotypic characterization of *E. coli* isolated from porcine sources.

Sample Number	Phylogroup	CH-Clonotype	ESBL Phenotype	AMR Phenotype [1]	AMR Genotype	Virulence Genes Array	Mutations QRDR [2] GyrA	Mutations QRDR ParC	Mutations QRDR ParE
1450	A	27-0	ESBL	AMP	*bla*TEM-1, *bla*CTX-M-1	*fimH1*, *faeG*, *astA*, *itcA*	w.t. [4]	w.t.	w.t.
3651	A	11-23	ESBL	AMP, CAZ, SXT	*bla*CTX-M-1, *sul2*, *dfr1*, *dfr17*	*fimH1*, *fimH2*, *fedA*, *estIa*, *estIb*	w.t.	w.t.	w.t.
3730	B1	23-31	n.a. [6]	AMP, SXT	n.dt. [5]	*fimH1*, *fimH2*, *eaeA*, *ent*	w.t.	w.t.	w.t.
4245	A	11-23	n.a.	NR	n.dt.	*fimH1*, *fimH2*, *faeG*, *estIa*, *estIb*	w.t.	w.t.	w.t.
4268	A	27-0	n.a.	TET	*tet*(A)	*fimH1*, *faeG*, *astA*, *itcA*	n.d.	n.d.	n.d.
101_76	A	27-0	n.a.	AMP, PIP, SXT	*sul2*, *dfr1*	*fimH1*, *faeG*, *astA*, *itcA*, *hlyA*	w.t.	w.t.	w.t.
103_78	C	11-24	ESBL	AMP, PIP, TET, FEP	*bla*CTX-M-1, *tet*(A), *tet*(B)	*fimH1*, *estIa*	w.t.	w.t.	w.t.
104_79	B1	6-31	n.a.	TET	*tet(*A)	*fimH1*	n.d. [3]	n.d.	n.d.

105_80	B1	1367-86	n.a.	AMP, TET, SXT, CIP	*tet*(A), *tet*(B), *sul2*, *dfr1*, *dfr12*	*fimH1*, *fimH2*	*gyrA S83L*, *gyrA S83A*, *gyrA D87N*	w.t.	w.t.
106_81	clade 1	11-53	n.a.	AMP, TET	*tet*(A)	*fimH1*, *fimH2*, *astA*, *aidA*	n.d.	n.d.	n.d.
107_82	A	11-23	n.a.	TET	*tet*(B)	*fimH1*, *fimH2*, *aidA*	n.d.	n.d.	n.d.
108_83	A	27-0	n.a.	NR	n.dt.	*fimH1*, *faeG*, *astA*, *itcA*	n.d.	n.d.	n.d.
109_84	B1	4-0	n.a.	AMP	*bla*TEM-1	*fimH1*, *fimH2*, *iucD*, *papC*	n.d.	n.d.	n.d.
17_1	A	7-54	n.a.	NR	n.dt.	*fimH1*, *fimH2*	n.d.	n.d.	n.d.
18_2	D	28-65	n.a.	AMP, PIP, TET, TOB	*tet*(B), *aadA1*	*fimH1*, *fimH2*, *fedA*, *astA*, *itcA*, *estIb*	w.t.	w.t.	w.t.
19_3	C	11-54	ESBL	AMP, PIP, TET, CTX, CHL, SXT, CIP	*bla*TEM-1, *bla*CTX-M-1, *tet*(B), *catA*, *sul2*, *dfr17*, *qnrS*	*fimH1*, *fimH2*, *iucD*, *papC*	*gyrA S83L*, *gyrA D87N*	*parC A56T*	w.t.

Sample Number	**Phylogroup**	**CH-Clonotype**	**ESBL Phenotype**	**AMR Phenotype [1]**	**AMR Genotype**	**Virulence Genes Array**	**Mutations QRDR [2] GyrA**	**Mutations QRDR ParC**	**Mutations QRDR ParE**
20_4	A	11-23	n.a.	TET, SXT	*tet*(A), *tet*(B), *sul2*, *dfr1*	*fimH1*	n.d.	n.d.	n.d.
21_5	A	29-32	n.a.	NR	n.dt.	*fimH1*, *fimH2*	n.d.	n.d.	n.d.
22_6	A	11-53	ESBL	AMP, PIP, CAZ	*bla*TEM-1, *bla*CTX-M-1	*fimH1*, *fimH2*	n.d.	n.d.	n.d.
23_7	G	45-97	n.a.	AMP, TET, SXT	*tet*(A), *sul1*, *sul*2, *dfr*17	*fimH1*, *fimH2*, *iucD*, *papC*, *pic*	w.t.	w.t.	w.t.
24_8	B2	40-22	n.a.	AMP, PIP, TET, CTX, FEP, ATM	*tet*(A)	*fimH1*, *fimH2*, *iucD*, *papC*	w.t.	w.t.	w.t.
25_9	A	11-41	n.a.	AMP, TET	n.dt.	*fimH1*	n.d.	n.d.	n.d.
27_11	B1	4-31	n.a.	AMP	*bla*TEM-1	*fimH1*, *fimH2*	n.d.	n.d.	n.d.
28_12	F	88-58	n.a.	AMP, TET, GEN, SXT, CIP	*tet*(B), *cmlA*, *sul2*, *dfr*17	*fimH1*, *fimH2*, *astA*, *faeG*, *iucD*, *papC*	*gyrA S83L*, *gyrA D87N*	*parC E84G*	*parE I355T*
29_13	F	88-54	n.a.	TET, GEN, SXT, CIP	*tet*(B), *sul*2, *dfr*17	*fimH1*, *fimH2*, *astA*, *iucD*, *papC*	*gyrA S83L*, *gyrA D87N*	*parC E84G*	*parE I355T*
26_10	B1	19-86	AmpC	AMP, TET, CAZ	*bla*CMY-2, *bla*TEM-1	*fimH1*	n.d.	n.d.	n.d.
2945_3	C	4-54	n.a.	NR	n.dt.	*fimH1*, *astA*, *estIa*	w.t.	w.t.	w.t.
30_14	A	7-54	n.a.	AMP, TET	*bla*TEM-1, *tet*(A), *tet*(B)	*fimH1*, *fimH2*	n.d.	n.d.	n.d.
32_16	C	4-35	ESBL	AMP, PIP, TET	*bla*TEM-1, *tet*(A)	*fimH1*, *fimH2*, *iucD*, *papC*	n.d.	n.d.	n.d.
33_17	A	7-0	n.a.	TET	*tet*(A)	*fimH1*	n.d.	n.d.	n.d.

34_18	C	4-24	n.a.	AMP, PIP, CTX, FEP, GEN, CHL, CIP, ATM	*bla*TEM-1, *tet*(A), *tet* (B), *aadA1*, *florF*	*fimH1*, *fimH2*, *iucD*, *papC*	*gyrA S83L*, *gyrA D87N*	w.t.	w.t.
35_19	A	11-54	ESBL	AMP, TET, CAZ, SXT, CIP	*tet*(A), *sul3*, *dfr1*, *qnrS*	*fimH1*, *fimH2*, *fanA*, *estIa*	*gyrA S83L*, *gyrA D87N*	w.t.	w.t.
36_20	D	11-32	n.a.	AMP, PIP, TET	*bla*TEM-1, *tet*(A)	*fimH1*, *fimH2*, *iucD*, *papC*	n.d.	n.d.	n.d.
37_21	A	11-27	n.a.	NR	n.dt.	*fimH1*, *fimH2*, *astA*, *estIa*, *stxa2*	w.t.	w.t.	w.t.
38_22	B1	65-32	n.a.	AMP, TET, SXT, CIP	*bla*TEM-1, *tet*(B), *sul2*, *dfr17*	*fimH1*, *fimH2*, *iucD*, *papC*	*gyrA S83L*, *gyrA D87N*	w.t.	w.t.
39_23	C	11-23	n.a.	AMP, TET, CHL, SXT, CIP	*bla*TEM-1, *tet*(A), *cml*-A1, *sul3*, *florF*, *dfr1*, *dfr12*	*fimH1*, *fimH2*	*gyrA S83L*, *gyrA D87N*	w.t.	w.t.
40_24	B1	23-158	n.a.	TET	*tet*(B)	*fimH1*, *fimH2*, *fasA*, *estIa*	n.d.	n.d.	n.d.
40541_1	C	4-27	n.a.	NR	n.dt.	*fimH1*, *fimH2*, *iucD*, *papC*	n.d.	n.d.	n.d.
40541_2	A	4-27	n.a.	AMP, PIP, TET	*bla*TEM-1, *tet*(A)	*fimH1*, *faeG*, *astA*, *itcA*	w.t.	w.t.	w.t.
41_25	B1	4-57	n.a.	NR	n.dt.	*fimH1*, *fimH2*, *hlyA*	n.d.	n.d.	n.d.

Sample Number	**Phylogroup**	**CH-Clonotype**	**ESBL Phenotype**	**AMR Phenotype** [1]	**AMR Genotype**	**Virulence Genes Array**	**Mutations QRDR** [2] **GyrA**	**Mutations QRDR ParC**	**Mutations QRDR ParE**
42_26	A	11-54	n.a.	AMP, TET	*bla*TEM-1, *tet*(B)	*fimH1*, *fimH2*, *hlyA*	n.d.	n.d.	n.d.
43_27	A	11-54	n.a.	AMP, TET	*bla*TEM-1, *tet*(A)	*fimH1*, *fimH2*	w.t.	w.t.	w.t.
4347_1	B1	579-0	n.a.	AMP, TET, CHL, GEN, TOB	*tet*(A), *aadA1*	*fimH1*, *estIa*, *fasA*	n.d.	n.d.	n.d.
4347_2	B1	579-0	n.a.	AMP, TET	*bla*TEM-1, *tet*(A)	*fimH1*, *estIa*, *fasA*	n.d.	n.d.	n.d.
4347_3	B1	579-0	n.a.	TET, FOF	*bla*TEM-1, *tet*(A)	*fimH1*, *estIa*, *fasA*	n.d.	n.d.	n.d.
44_28	C	4-39	ESBL	AMP, PIP, TET, CAZ, SXT	*bla*TEM-1, *tet*(A), *sul2*, *sul3*	*fimH1*, *fimH2*, *iucD*, *papC*	n.d.	n.d.	n.d.
448_1	A	27-0	ESBL	AMP, CAZ, FEP, ATM	*bla*TEM-1, *bla*CTX-M-1	*fimH1*, *faeG*, *astA*, *itcA*	w.t.	w.t.	w.t.
448_2	A	27-0	ESBL	AMP, PIP, CTX, FEP, ATM	*bla*TEM-1, *bla*CTX-M-1	*fimH1*, *faeG*, *astA*, *itcA*	n.d.	n.d.	n.d.
45_29	A	11-0	ESBL	AMP, TET, CAZ	*bla*TEM-1, *bla*CTX-M-1, *tet*(A)	*fimH1*, *fimH2*	n.d.	n.d.	n.d.

46_30	A	11-23	n.a.	TET	*tet*(A)	*fimH1*, *fimH2*, *aidA*, *stx2e*	w.t.	w.t.	w.t.
47_31	A	11-0	n.a.	AMP, TET, GEN, TOB, CIP	*tet*(B), *aac3*^{0}*-II*, *aac5-Ib-cr*	*fimH1*, *astA*, *iucD*, *papC*	*gyrA S83L, gyrA D87N*	w.t.	w.t.
48_32	A	11-25	ESBL	AMP, PIP, CTX	*bla*TEM-1, *bla*CTX-M-1	*fimH1*, *fimH2*	n.d.	n.d.	n.d.
49_33	B1	4-86	n.a.	AMP, TET	*tet*(A), *tet*(G)	*fimH1*, *fimH2*, *iucD*, *papC*	n.d.	n.d.	n.d.
50_34	B1	23-158	n.a.	TET	*tet*(A)	*fimH1*, *fimH2*, *pic*	n.d.	n.d.	n.d.
51_15	B1	19-32	n.a.	AMP, TET	*tet*(B)	*fimH1*	w.t.	w.t.	w.t.
566_1	D	28-41	n.a.	NR	n.dt.	*fimH1*, *fimH2*, *fedA*, *estIa*, *estIb*, *aidA*, *hlyA*, *stxa2*, *stx2e*	w.t.	w.t.	w.t.
566_2	D	28-41	n.a.	NR	n.dt.	*fimH1*, *fimH2*, *fedA*, *estIa*, *aidA*, *hlyA*, *stxa2*, *stx2e*	n.d.	n.d.	n.d.
566_3	D	28-41	n.a.	NR	n.dt.	*fimH1*, *fimH2*, *fedA*, *estIa*, *aidA*, *hlyA*, *stxa2*, *stx2e*	n.d.	n.d.	n.d.
60_35	A	11-54	n.a.	AMP, PIP, TET, CTX, CHL, SXT, CIP	*bla*TEM-1, *tet*(A), *sul1*, *sul2*, *sul3*, *dfr1*, *dfr12*, *dfr17*, *catA*, *cmlA1*	*fimH1*, *fimH2*	*gyrA S83L, gyrA D87N*	w.t.	w.t.

Sample Number	**Phylogroup**	**CH-Clonotype**	**ESBL Phenotype**	**AMR Phenotype** [1]	**AMR Genotype**	**Virulence Genes Array**	**Mutations QRDR** [2] **GyrA**	**Mutations QRDR ParC**	**Mutations QRDR ParE**
61_36	B1	19-38	n.a.	AMP, TET, GEN, SXT, CIP	*bla*TEM-1, *tet*(A), *tet*(B), *sul1*, *sul2*, *dfr*17, *aadA1*, *aadA5*, *qnrS*	*fimH1*, *fimH2*, *iucD*, *papC*	*gyrA S83L, gyrA D87N*	w.t.	w.t.
62_37	A	7-54	n.a.	AMP, TET	*bla*TEM-1, *tet*(A), *tet*(B)	*fimH1*, *fimH2*	*n.d.*	n.d.	n.d.
63_38	B1	7-31	n.a.	AMP, PIP, TET, CTX, CIP	*bla*TEM-1, *tet*(A), *qnr*S	*fimH1*, *fimH2*, *astA*	*gyrA S83L, gyrA D87N*	w.t.	w.t.
630_2	A	27-0	n.a.	NR	n.dt.	*fimH1*, *fimH2*, *faeG*, *astA*, *itcA*	n.d.	n.d.	n.d.
64_39	B2	52-5	ESBL	AMP, PIP, TET, CTX	*bla*TEM-1, *tet*(A)	*fimH1*, *fimH2*, *astA*, *papC*, *iucD*, *cnf1*	n.d.	n.d.	n.d.
65_40	A	11-0	ESBL	AMP, TET, SXT, CTX?, CAZ	*bla*TEM-1, *bla*CTX-M-1, *tet*(A), *tet*(B), *sul*2	*fimH1*	n.d.	n.d.	n.d.

66_41	B1	41-54	ESBL	AMP, PIP, TET, CTX, FEP, SXT, ATM	*bla*TEM-1, *bla*CTX-M-1, *tet*(A), *tet*(B), *sul2*, *dfr1*, *dfr12*	*fimH1*, *fimH2*	w.t.	w.t.	w.t.
67_42	A	27-0	n.a.	AMP	n.dt.	*fimH1*, *astA*, *faeG*, *itcA*, *hlyA*	w.t.	w.t.	w.t.
68_43	A	27-0	n.a.	AMP, PIP	n.dt.	*fimH1*, *astA*, *faeG*, *itcA*, *hlyA*	w.t.	w.t.	w.t.
69_44	A	41-38	n.a.	TET	*tet*(B)	*fimH1*, *fimH2*, *iucD*, *papC*	w.t.	w.t.	w.t.
70_45	B1	579-0	n.a.	AMP, CHL, SXT	*sul2*, *dfr1*, *catA*	*fimH1*, *estIa*, *fasA*	w.t.	w.t.	w.t.
71_46	A	11-54	n.a.	AMP, PIP	*bla*TEM-1	*fimH1*	n.d.	n.d.	n.d.
72_47	A	11-45	n.a.	NR	n.dt.	*fimH1*, *fimH2*, *astA*, *aidA*	n.d.	n.d.	n.d.
73_48	A	11-45	n.a.	CHL, SXT?	*cmlA1*	*fimH1*, *fimH2*, *astA*, *aidA*	w.t.	w.t.	w.t.
74_49	A	11-23	n.a.	AMP, TET	*bla*TEM-1, *tet*(A)	*fimH1*, *fimH2*	n.d.	n.d.	n.d.
75_50	A	11-24	n.a.	AMP, TET	*bla*TEM-1, *tet*(A), *tet*(B)	*fimH1*, *fimH2*	n.d.	n.d.	n.d.
76_51	A	27-0	n.a.	NR	n.dt.	*fimH1*, *faeG*, *astA*, *itcA*	w.t.	w.t.	w.t.
77_52	E	7-31	n.a.	NR	n.dt.	*fimH1*, *fimH2*	n.d.	n.d.	n.d.
78_53	A	11-23	n.a.	TET	*tet*(A)	*fimH1*, *fimH2*	n.d.	n.d.	n.d.
79_54	A	11-24	n.a.	TET	*tet*(A)	*fimH1*, *fimH2*	n.d.	n.d.	n.d.
80_55	A	11-54	n.a.	TET, CHL	*tet*(A), *cmlA1*	*fimH1*, *fimH2*	n.d.	n.d.	n.d.
81_56	A	4-24	n.a.	NR	n.dt.	*fimH1*, *fimH2*	n.d.	n.d.	n.d.
82_57	B1	6-289	n.a.	NR	n.dt.	*fimH1*, *fimH2*, *astA*, *fedA*, *aidA*, *stx2e*	w.t.	w.t.	w.t.
83_58	B2	11-25	n.a.	AMP, PIP, FOF	*bla*TEM-1, *fosB*	*fimH1*, *cnf1*	n.d.	n.d.	n.d.

Sample Number	**Phylogroup**	**CH-Clonotype**	**ESBL Phenotype**	**AMR Phenotype [1]**	**AMR Genotype**	**Virulence Genes Array**	**Mutations QRDR [2] GyrA**	**Mutations QRDR ParC**	**Mutations QRDR ParE**
84_59	B1	23-158	ESBL	AMP, CTX, FEP	*bla*TEM-1 *bla*CTX-M-1	*fimH1*, *fimH2*	n.d.	n.d.	n.d.
85_60	A	4-27	ESBL	AMP, PIP, TET, CTX, CAZ, FEP	*bla*TEM-1, *tet*(B)	*fimH1*, *iucD*, *papC*	n.d.	n.d.	n.d.
86_61	B1	41-0	n.a.	NR	n.dt.	*fimH1*, *fimH2*, *iucD*, *papC*	n.d.	n.d.	n.d.
87_62	C	11-35	ESBL	AMP, PIP	*bla*TEM-1	*fimH1*, *iucD*, *papC*	n.d.	n.d.	n.d.
88_63	A	11-25	n.a.	AMP, CTX	*bla*TEM-1, *bla*CTX-M-1	*fimH1*, *fimH2*	n.d.	n.d.	n.d.

89_64	A	27-0	n.a.	TET, SXT, CIP	*tet*(A), *sul*[1], *sul2*, *dfr1*	*fimH*, *astA*, *iucD*, *papC*	*gyrA S83L, gyrA D87N*	w.t.	w.t.
90_65	A	27-0	n.a.	TET, GEN, TOB,	*tet*(A), *aadA1*, *aadA2*, *aadA5*	*fimH1*, *faeG*, *astA*, *itcA*	n.d.	n.d.	n.d.
91_66	A	11-25	n.a.	NR		*fimH1*, *fimH2*	n.d.	n.d.	n.d.
92_67	A	11-398	ESBL	AMP, TET	*bla*TEM-1, *tet*(B)	*fimH1*, *fimH2*	n.d.	n.d.	n.d.
93_68	B1	4-32	ESBL	AMP, TET, CHL, CIP	*bla*TEM-1, *tet*(C), *catA*, *florF*, *cmlA*	*fimH1*, *fimH2*, *iucD*, *papC*	*gyrA S83L, gyrA D87N*	w.t.	w.t.
94_69	B1	41_86	n.a.	AMP, TET, SXT	*bla*TEM-1, *tet*(A), *sul1*, *sul2*, *aadA1*	*fimH1*, *fimH2*	w.t.	w.t.	w.t.
95_70	A	11-34	ESBL	AMP, PIP, TET	*bla*TEM-1, *bla*CTX-M-1, *tet*(A), *tet*(B)	*fimH1*, *fimH2*	n.d.	n.d.	n.d.
96_71	B1	4-27	n.a.	NR	n.dt.	*fimH1*, *iucD*, *papC*	n.d.	n.d.	n.d.
97_72	A	27-54	n.a.	TET	*tet*(A)	*fimH1*, *fimH2*	n.d.	n.d.	n.d.
98_73	A	7-54	n.a.	NR	n.dt.	*fimH1*, *fimH2*, *astA*, *aidA*, *bfpB*	w.t.	w.t.	w.t.
99_74	B2	40-22	n.a.	AMP, PIP, TET, SXT	*tet*(A), *dfr1*, *dfr17*	*fimH1*, *fimH2*, *papC*, *iucD*, *cnf1*	w.t.	w.t.	w.t.
3835_2	B1	4-440	n.a.	AMP, PIP, TET, CHL, SXT, COL	*mcr1*	*fimH1*, *fimH2*, *astA*, *eaeA*, *ent*, *escV*, *hlyA*	w.t.	w.t.	w.t.
3835_3	A	11-54	n.a.	AMP, PIP, TET, CHL, SXT, COL	*mcr1*	*fimH1*, *fimH2*	w.t.	w.t.	w.t.
3835_4	A	11-54	n.a.	AMP, PIP, TET, CHL, SXT, COL	*mcr1*	*fimH1*, *fimH2*	w.t.	w.t.	w.t.

Table 2. Characterization of whole-genome-sequenced porcine *E. coli*.

Isolate	**Phylogroup**	**CH-Clonotype**	**Serotype** [1]	**SequenceType**	**ESBL** [6]	**AMR Phenotype** [2]	**WGS AMR Genes**	**WGS VAG**	**QRDR** [4] ***GyrA*** [3]	**QRDR** [4] ***ParC*** [3]	**QRDR** [4] ***ParE*** [3]
1450	A	27-0	O_{NT}:H10	clustered 100	ESBL	AMP	*bla*TEM-1B *, *bla*CTX-M-1 *, *mdfA*, *mphA*	*faeG* *, *astA* *, *capU*, ***cba*** [5], ***cia***, *cma* *, *gad*, *iha*, *ltcA* *, *stb* *, *terC*, ***traT***	w.t.	w.t.	w.t.
3651	A	11-23	O_{NT}:H32	10	ESBL	AMP, CAZ, SXT	***bla*CTX-M-1**, ***sul2***, ***dfrA17***, ***aadA5***, *mdfA*	***cib***, ***fedA***, ***fedF***, ***gad***, *iss*, *ompT*, *sta1*, *stb*, *terC*, ***traT***	w.t.	w.t.	w.t.
3730	B1	23-31	O_{NT}:H21	56	n.a. [7]	AMP, SXT	***bla*TEM-1B**, *sul1* *, *sul2* *, *dfrA1* *, *aadA1* *, *aph(3")-Ib* *, *aph(6)-Id* *, *mdfA*, ***mphB***	***cma***, ***cvaC***, *gad*, ***hlyF***, ***iroN***, ***iss***, *lpfA*, *ompT*, *sitA*, *terC*, ***traT***	w.t.	w.t.	w.t.

[1] Abbreviations: AMC, amoxicillin/clavulanate; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; CFZ, cefazolin; CTX, cefotaxime; FOF, fosfomycin; GEN, gentamicin; PIP, piperacillin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TOB, tobramycin; COL, colistin; NR, not resistant. [2] QRDR: quinolone-resistance-determining region. [3] n.d., not done. [4] w.t., wild type. [5] n.dt., none detected using the primer-set of this study. [6] n.a., not applicable.

4245	A	11-23	O_{NT}:H26	1112	n.a.	NR	*sul1* *, *aadA1* *	***faeG***, ***cea***, ***cib***, *gad*, ***sepA*** *, *sta1* *, *stb*, *terC*, ***traT***	w.t.	w.t.	w.t.
101_76	A	27-0	O_{NT}:H10	clustered 100	n.a.	AMP, PIP, SXT	*bla*TEM-1C *, *mdfA*, *sul2* *, *dfrA1* *, *qnrD1* *, *aph(3")-Ib* *, *aph(6)-Id* *	***faeG***, ***astA,*** *capU*, *gad*, *iha*, *stb*, *terC*, *traT*	w.t.	w.t.	w.t.
103_78	C	11-24	O_{NT}:H12	10	ESBL	AMP, PIP, TET, FEP	***bla*CTX-M-1**, *tet*(B) *, *sul1* *, ***mdfA***, *mphA*, *aadA1* *,	***cia***, *hra*, *iha*, *iroN*, *ompT*, ***papC***, *terC*, ***traT***	w.t.	w.t.	w.t.
18_2	D	28-65	O108:H4	42	n.a.	AMP, PIP, TET, TOB	*bla*TEM-1B *, *tet*(B) *, *aac(3)-IV* *, *aadA1*, *aph(3")-Ib* *, *aph(6)-Id* *, *aph(4)-Ia* *, *mdfA*	*air*, *astA*, *chuA*, ***fedA***, ***fedF***, *hra*, *iha*, *iss*, *lpfA*, *ltcA*, *neuC*, *ompT*, *stb*, *terC*, ***traT***	w.t.	w.t.	w.t.
19_3	C	11-54	O_{NT}:H10	744	ESBL	AMP, PIP, TET, CTX, CHL, SXT, CIP	*tet*(B) *, ***sul1***, *sul2* *,***dfrA17***, *aph(3^0)-Ia* *, *aph*(3")-Ib *, *aph(6)-Id* *, ***aadA5***, *cat*A1 *, *mdfA*	***cba***, ***cia***, *cma* *, *cvaC*, *etsC* *, *gad*, ***hlyF***, *iroN*, ***iss***, ***iucC***, ***iutA***, *mchF*, *ompT* *, *sitA*, *terC*, ***traT***, *tsh* *	*gyrA S83L*, *gyrA D87N*	*par*C A56T	w.t.
23_7	G	45-97	O_{NT}:H4	117	n.a.	AMP, TET, SXT	***bla*TEM-1B**, ***sul1***, *sul2* *, *tet*(A) *, ***dfrA17***, *mdfA*, ***mphA***, *aph(3")-Ib* *, *aph(6)-Id* *, ***aadA5***	*cea*, *chuA*, *fyuA*, *gad*, *hlyE*, ***hlyF***, *ireA*, *iroN*, *irp2*, *iss*, ***iucC***, ***iutA***, *katP*, *lpfA*, ***ompT***, *pic*, ***sitA***, *terC*, ***traT***, *vat*	w.t.	w.t.	w.t.
24_8	B2	40-22	025:H4	131	n.a.	AMP, PIP, TET, CTX, FEP, ATM	***bla*TEM-1C**, *bla*CTX-M-1 *, *tet*(A) *, ***aph(3^0)-Ia***, *mphA* *, *mdfA*, *qnrS1* *	*chuA*, ***cia***, *cvaC* *, *etsC*, *fyuA*, *gad*, ***hlyF***, *hra*, *ibeA*, *iroN*, *irp2*, *iss*, ***iucC***, ***iutA***, *kpsE*, *kpsMII*, *mchF* *, ***ompT***, *papA-F48*, *papC*, *sitA*, *terC*, ***traT***, *usp*, *yfcV*	w.t.	w.t.	w.t.

Isolate	**Phylogroup**	**CH-Clonotype**	**Serotype [1]**	**SequenceType**	**ESBL [6]**	**AMR Phenotype [2]**	**WGS AMR Genes**	**WGS VAG**	**QRDR [4] *GyrA* [3]**	**QRDR [4] *ParC* [3]**	**QRDR [4] *ParE* [3]**
28_12	F	88-58	O_{NT}:H34	354	n.a.	AMP, TET, GEN, SXT, CIP	***bla*TEM-1B**, *sul2* *, *tet*(B) *, *dfrA17* *, *aph(3")-Ib* *, *aph(6)-Id* *, *aac(3)-IId*, *aph(3^0)-Ia* *, *mdfA*	*air*, *astA*, *chuA*, *eilA*, *gad*, *hra*, *ibeA*, ***iucC***, ***iutA***, *kpsE*, *kpsMII_K5*, *lpfA*, ***sitA***, *terC*, *usp*, *yfcV*	*gyrA S83L*, *gyrA D87N*	*parC E84G*	*parE I355T*

29_13	F	88-54	O_{NT}:H34	354	n.a.	TET, GEN, SXT, CIP	*tet*(B) *, *sul*2 *, ***dfrA17***, *aph(3")-Ib **, aac(3)-IId*, *aph(6)-Id **, *mdfA*	*air*, *astA*, *chuA*, *eilA*, *gad*, *hra*, *ibeA*, ***iucC***, ***iutA***, *kpsE*, ***kpsMII_K5***, *lpfA*, ***sitA***, *terC*, *usp*, *yfcV*	*gyrA S83L*, *gyrA D87N*	*parC E84G*	*parE I355T*
2945_3	C	4-54	O8:H17	23	n.a.	NR	*mdfA*	*asta*, *cia **, *fanA*, *fyuA*, *gad*, *irp2*, *iss*, *lpfA*, ***mcbA***, *ompT*, ***sepA***, *terC*, ***traT***	w.t.	w.t.	w.t.
35_19	A	11-54	O_{NT}:H9	10	ESBL	AMP, TET, CAZ, SXT, CIP	*bla*TEM-52B *, ***tet*(B)**, *sul1 **, *dfrA1 **,***aph(3")Ib***, *aadA1*, ***aph(6)-Id***, *mdfA*	***cia***, ***cib***, ***fanA***, *gad*, *iss*, *terC*, *traT **	*gyrA S83L*, *gyrA D87N*	w.t.	w.t.
37_21	A	11-27	O_{NT}:H16	neuer ST	n.a.	NR	*mdfA*	***astA***, *gad*, *iha*, *iss*, *lpfA*, ***sepA***, ***sta1***, ***stb***, *stx2A*, *stx2B*, *terC*, *traT*, *stx2*	w.t.	w.t.	w.t.
40541_2	A	4-27	n.t.	100	n.a.	AMP, PIP, TET	***bla*TEM-1B**, *tet*(A) *, *sul*2 *, *dfrA14 **, *mdfA*, *aph(3")-Ib **, *aph(6)-Id **	*faeG **, *astA **, *capU*, ***cib***, *gad*, *iha*, *ltcA **, *stb **, *terC*, *traT*	w.t.	w.t.	w.t.
4347_1	B1	579_0	O64:H-	6404	n.a.	AMP, TET, CHL, GEN, TOB	*bla*TEM-1B *, ***tet*(A)**, ***sul1***, *qnrS1*, *aph(3")-Ib*, *aph(6)-Id*, *aph(4)-Ia **, *aac(3)-IV **, ***aadA1***, *mdfA*, *catA1 **	***cba***, ***cea***, *cma*, *fasA*, *gad*, *iss*, *lpfA*, *ompT*, *terC*	w.t.	w.t.	w.t.
448_1	A	27-0	O_{NT}:H10	clustered 100	ESBL	AMP, CAZ, FEP, ATM	***bla*CTX-M-1 ***, *bla*TEM-1B, *mphA **, *mdfA*	*faeG **, *astA*, *capU*, ***cba***, ***cia***, *cma*, *gad*, *iha*, *ltcA*, *stb*, *terC*, ***traT***	w.t.	w.t.	w.t.
46_30	A	11-23	O142:H27	neu icd	n.a.	TET	*tet*(A), *mdfA*	*stx2*, ***sepA***, *stx2A*, *stx2B*, *terC*, ***traT***	w.t.	w.t.	w.t.
51_15	B1	19-32	O_{NT} H49	1079	n.a.	AMP, TET	*bla*TEM-1B *, *tet*(B) *, *mdfA*, *aph(3")-Ib*, *aph(6)-Id*	*gad*, *lpfA*, *terC*	w.t.	w.t.	w.t.
566_1	D	28-41	O138:H14	760	n.a.	NR	*mdfA*	*stx2*, *chuA*, ***fedA***, ***fedF***, *gad*, *hra*, *iha*, *iss*, *ompT*, *sta1*, *stb*, *stx2A*, *stx2B*, *terC*, ***traT***	w.t.	w.t.	w.t.

Isolate	**Phylogroup**	**CH-Clonotype**	**Serotype [1]**	**SequenceType**	**ESBL [6]**	**AMR Phenotype [2]**	**WGS AMR Genes**	**WGS VAG**	**QRDR [4] *GyrA* [3]**	**QRDR [4] *ParC* [3]**	**QRDR [4] *ParE* [3]**

66_41	B1	41-54	O88:H21	101	ESBL	AMP, PIP, TET, CTX, FEP, SXT, ATM	*bla*TEM-1B *, **blaCTX-M-1**, *tet*(B) *, *dfrA1* *, *aadA1* *, *qnrS1* *, *mdfA*, ***mphA***	*gad*, *hra*, *iss*, *lpfA*, *ompT*, *terC*	w.t.	w.t.	w.t.
67_42	A	27-0	O_{NT}:H10	clustered 100	n.a.	AMP	***bla*TEM-1B**, *mdfA*	***faeG***, ***astA***, ***capU***, ***cba***, ***cia***, *cma*, *gad*, *iha*, ***ltcA***, *stb*, *terC*, ***traT***	w.t.	w.t.	w.t.
68_43	A	27-0	O_{NT}:H10	clustered 100	n.a.	AMP, PIP	***bla*TEM-1B**, *mdfA*	*faeG* *, *astA*, *capU*, ***cba***, ***cia***, *cma*, *gad*, *iha*, *ltcA*, *stb*, *terC traT* *	w.t.	w.t.	w.t.
69_44	A	41-38	O_{NT}:H21	101	n.a.	TET	*tet*(B) *, *mdfA*, *aph(3")-Ib*, *aph(6)-Id*	***cia***, *cvaC* *, *etsC*, *gad*, ***hlyF***, *iroN*, *iss*, ***iucC***, ***iutA***, *lpfA*, *ompT*, ***sitA***, *terC*, ***traT***	w.t.	w.t.	w.t.
70_45	B1	579-0	n.t.	6404	n.a.	AMP, CHL, SXT	***bla*TEM-1B**, ***sul1***, *sul2* *, *dfrA1* *, ***aadA1***, *aph(3")-Ib* *, *aph(6)-Id* *, *mdfA*, ***catA1***	***cba***, ***cea***, ***cia***, *cma*, *fasA*, *gad*, *iss*, *lpfA*, *ompT*, *terC*, *traT*	w.t.	w.t.	w.t.
73_48	A	11-45	O_{NT}:H6	10	n.a.	CHL, SXT	***cmlA1***,***sul3***, ***dfrA12***, ***aadA2***, ***aadA1***, *mdfA*	***astA***, *gad*, *stb*, *terC*	w.t.	w.t.	w.t.
76_51	A	27-0	O_{NT}:H10	100	n.a.	NR	*sul2* *, *mdfA*, *aph(6)-Id* *, *aph(3")-Ib* *	*faeG* *, ***astA***, *capU*, *cba*, *cma*, *gad*, *iha*, *terC*, ***traT***	w.t.	w.t.	w.t.
82_57	B1	6-289	O121:H10	641	n.a.	NR	*mdfA*	***stx2***, ***astA***, ***fedA***, ***fedF***, *gad*, *lpfA*, ***sepA***, ***stx2A***, ***stx2B***, *terC*, ***traT***	w.t.	w.t.	w.t.
94_69	B1	41-86	O82:H8	6365	n.a.	AMP, TET, SXT	*mdfA*, ***sul1***, ***tet*(C)**, ***aadA1***	***cea***, *cnf1*, *cvaC*, *etsC* *, *gad*, *hlyF* *, *hra*, *iroN*, *iss*, ***iucC***, ***iutA***, *lpfA*, *mchF*, *ompT* *, *papA-F1651A*, *papC*, ***sitA***, *terC*, ***traT***, *tsh*	w.t.	w.t.	w.t.
98_73	A	7-54	O_{NT}:H10	neu icd	n.a.	NR	*mdfA*	***astA***, *fyuA*, *irp2*, *papC*, ***stb***, *terC*, ***traT***	w.t.	w.t.	w.t.
99_74	B2	40-22	O25:H4	131	n.a.	AMP, PIP, TET, SXT	***bla*TEM-1C**, ***aadA1***, *mdfA*, *tet*(A) *, ***dfrA1***, ***sul3***	***cea***, *chuA*, ***cia***, *cnf1*, *cvaC*, *etsC*, *fyuA*, *gad*, ***hlyF***, *hra*, *ibeA*, *iroN*, *irp2*, *iss*, ***iucC***, ***iutA***, *kpsE*, *kpsMII_K5*, *mchF*, ***ompT***, *papA_F14*, *papC*, ***sitA***, *terC*, ***traT***, *usp*, *yfcV*	w.t.	w.t.	w.t.

Isolate	Phylogroup	CH-Clonotype	Serotype [1]	SequenceType	ESBL [6]	AMR Phenotype [2]	WGS AMR Genes	WGS VAG	QRDR [4] *GyrA* [3]	QRDR [4] *ParC* [3]	QRDR [4] *ParE* [3]
3835_2	B1	4-440	O26:H11	88	n.a.	AMP, PIP, TET, CHL, SXT, COL	*aadA1* *, *aadA2* *, *cmlA1* *, *mcr-1.1* *, *tet*(A) *, ***tet*(M)**, *mefB* *, *mdfA*, *dfrA12* *, **bla**$_{\text{TEM-1B}}$, *sul3* *	*astA*, ***cif***, *eaE*, *efa1*, ***ehxA***, ***espP***, *espA*, *espB*, *espF*, *espJ*, *espP*, *fyuA*, *gad*, *iha*, *irp2*, *iss*, ***katP***, *lpfA*, *nleA*, *nleB*, *ompT*, *terC*, *tir*, *traT* *	w.t.	w.t.	w.t.
3835_3	A	11-54	O2:H2	10	n.a.	AMP, PIP, TET, CHL, SXT, COL	*tet*(A) *, ***sul3***, ***aph(3")-Ib***, *aadA2* *, ***aph(6)-Id***, *mdf*(A), *dfrA12* *, *cmlA1* *, ***mcr-1.1***, ***bla***$_{\text{TEM-1D}}$	***cea***, *cvaC*, *gad*, *hra*, *iha*, *iss*, ***katP***, *mchF*, *terC*, *traT* *	w.t.	w.t.	w.t.
3835_4	A	11-54	O2:H2	10	n.a.	AMP, PIP, TET, CHL, SXT, COL	*tet*(A) *, *sul3* *, ***aph(3")-Ib***, ***aadA2***, ***aph(6)-Id***, *mdf*(A),***dfrA12***, ***cmlA1***, ***mcr-1.1***, ***bla***$_{\text{TEM-1D}}$	***cea***, *cvaC*, *gad*, *hra*, *iha*, *iss*, ***katP***, *mchF*, *terC*, ***traT***	w.t.	w.t.	w.t.

[1] n.t., not typeable. [2] Abbreviations: AMC, amoxicillin/clavulanate; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin;

CTX, cefotaxime; FOF, fosfomycin; GEN, gentamicin; PIP, piperacillin; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TOB, tobramycin; COL, colistin. [3] w.t., wild type. [4] QRDR: quinolone-resistance-determining region; w.t., wild type. [5] bold letters: plasmid predicted by mlplasmids. [6] ESBL: Extended-spectrum β-lactamase. [7] n.a., not applicable. * potentially plasmid-encoded as deduced from BLASTn analyses.

3.2. Characterization of Genotypic Antibiotic Resistance

In 13.71% of the isolates, genes from the *bla*$_{\text{CTX}}$ family were detected alone or combined with other *bla* genes. One of the isolates displayed an AmpC phenotype and carried a *bla*$_{\text{CMY-2}}$ gene. The most prevalent β-lactamase genes detected were *bla*$_{\text{TEM-1}}$ (56.00%) followed by *bla*$_{\text{CTX M-1}}$ (13.71%). Three isolates carried the mobile colistin resistance gene *mcr-1.1*.

The *gyrA* and *parC* sequences of 13.72% ciprofloxacin-resistant isolates were analyzed and revealed mutations that resulted in the following amino acid substitutions: 10.78% of the isolates had a Ser83Leu, one isolate a Ser83Ala, and another 10.78% of isolates an Asp87Asn substitution in *gyrA*, while in *parC* 11 isolates displayed a Ser80Ile, 1.96% of the isolates showed Glu84Gly mutation while one isolate revealed a Cys56Thr substitution. A total of 1.96% of all isolates had an Ile355Thr mutation in *parE*. Results are listed in Table 1.

3.3. Biocide Susceptibility Testing

The obtained MIC values of all tested biocides against ATCC strains, including *E. coli*

ATCC 10,536, were in the acceptable susceptibility. MIC values of benzalkonium chloride

(BAC) for all clinical *E. coli* isolates ranged from 0.0005% to 0.002%. The obtained BAC MIC values were 0.0005% for 1.9% of isolates (2/104), 0.001% for 54.7% (59/104) of isolates, and 0.002% for 41.3% (43/104) of isolates. Chlorhexidine (CHX) MIC values comprised seven dilutions steps from 0.00003% to 0.002%. In comparison to BAC with a unimodal distribution, a bimodal MIC distribution was seen for CHX. This bimodal distribution might point towards a possibly acquired resistance property for the isolates with CHX MICs of 0.00025%. For glutaraldehyde (GLU), unimodal MIC distribution comprising five dilution steps (0.03% to 0.5%) was observed. Except for one isolate with an MIC of <1%, the remaining isolates had isopropanol (ISO) MICs from 2% to 10%. The results of the biocide susceptibility testing of *E. coli* are shown in Supplementary Materials Tables S1 and S2.

3.4. E. coli Phylotyping

Among all *E. coli* isolates, the most dominant phylogenetic group was A (50.98%), followed by B1 (25.48%), while the remaining belonged to C (8.81%), D (5.87%), B2 (3.91%), F (1.95%), E, G, and clade 1 (each 0.97%). Results of *E. coli* phylotyping are shown in Tables 1 and 2 and Supplementary Materials Table S3.

3.5. E. coli Clonotyping

The *fumC* and *fimH* (CH) typing divided all isolates into 51 distinct CH clonotypes and revealed the clonal relatedness of 12 isolates (CH27-0), 9 isolates (CH11-54) and 8 isolates (CH11-23). *E. coli*-predicted CH clonotype CH40-24 was clearly determined in isolates 24_8 and 99_74. The relatedness of isolates is visualized in Figure 1.

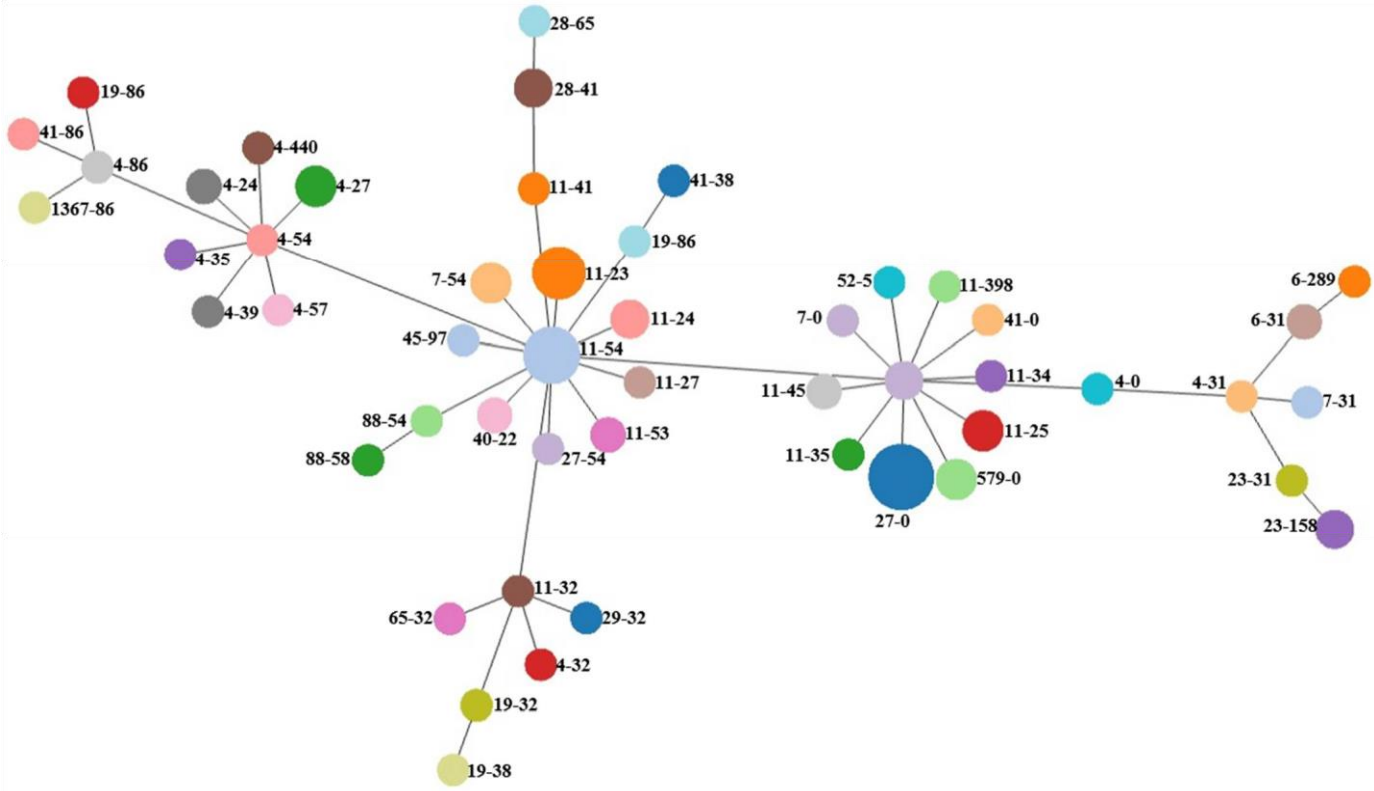

Figure 1. goeBURST diagram for the CH clonotyping dataset of *E. coli* isolates. An eBURST diagram was calculated using PHYLOViZ with the goeBURST algorithm. *E. coli* isolates were grouped according to their CH profiles.

3.6. Whole-Genome Sequencing (WGS) of Selected E. coli Isolates

In our study, 35 isolates were analyzed by whole-genome sequencing (WGS). WGS revealed a total of 16 distinct STs. The most common sequence type was ST10 (*n* = 6), which clustered together by cgMLST. Further sequence types were ST100 (*n* = 5), ST354, ST131

(*n* = 2 each), and singletons ST6404, ST6365, ST1112, ST1079, ST760, ST744, ST641, ST117, ST101, ST56, ST42, and ST23. New sequence types could be obtained in three isolates: ST12008 (37_21), ST12009 (46_30), ST12010 (98_73).

The WGS analysis revealed 12 different serogenotypes (WGS-predicted serotypes). The remaining 23 strains were O-non-typeable with 11 different H types. Three isolates could not be assigned to a known serotype. Isolates belonging to O25:H4 were detected in two cases. Three isolates carrying the gene *stx2e* could be assigned to serotype O138:H14.

Another *stx2*-carrying isolate belonged to serotype O121:H10.

In total, 6 out of 35 Shiga toxin-producing *E. coli* were detected in the present study carrying the genes *stx2*, *stx2e*, *stx2A* and *stx2B*.

Two out of 35 isolates belonged to the successful evolutionary line ST131 and could be assigned to phylogroup B2 (*fimH*22). Isolate 24_8 was a $bla_{\text{TEM-1C}}$ and $bla_{\text{CTX-M-1}}$-producing ESBL *E. coli* whereas isolate 99_74 produced only $bla_{\text{TEM-1C}}$. Both strains revealed mutations in the QRDR of *gyrA* and *parC* and were multidrug-resistant. Virulence potential for both strains was inferred by the detection of multiple VAGs determining the UPEC pathotype. Virulence profile similarity among the two isolates was high and the types of virulence genes presented in these strains were coding for adhesins, toxins, siderophores, hemolysins, and protectins.

PlasmidFinder was used for the analysis of WGS data and revealed the presence of the plasmid replicons IncFIB(AP001918), IncFIC(FII), IncHI2, IncHI2A, IncX1, IncFII, IncN, IncY, IncI1-I(Alpha), IncFIA, IncQ1, p0111, IncFII(pHN7A8), Col(MG828), IncR, IncFIB(H89-PhagePlasmid), IncFII(29), IncFII(pCoo), Col156, IncFII(pSE11), IncI2(Delta), IncI2, IncFII(pRSB107), Col440II, ColpVC, IncX4 and IncB/O/K/Z. IncX4 was identified as the replicon of all *mcr-1*-carrying plasmids. IncFIB(AP001918) plasmids were predominant (27 of 35) and carried the VAGs *ompT*, *hlyF*, *cia*, and *etsC*, followed by IncI1-I(Alpha) plasmids carrying *cia* and $bla_{\text{CTX-M-1}}$ and the IncX1 plasmids carrying $bla_{\text{TEM-1B}}$. IncFII and IncFII(pCoo) carried *traT*. The full list of VAG and AMR genes and their predicted plasmid probability are shown in Table 1, Supplementary Materials Tables S3–S6.

3.7. E. coli Pathotyping

All isolates carrying VAGs and VAGs related to pathogenic *E. coli* subtypes were frequently detected. A total of 30 genes were screened by using microarray-based diagnostics. The adhesion gene *fimH* was present in all but one isolate and therefore was the most frequent gene of the adhesins category. The iron acquisition gene *iucD* was found in 24 isolates and was always represented together with the fimbrial gene *papC*. Among toxin-encoding genes, *astA* was the most predominant (*n* = 27) gene, followed by *itcA* (*n* = 13). The shigatoxin *stx2e* gene was detected in five isolates and the gene *hlyA* (*n* = 9) occurred more often than the *cnf1* gene (*n* = 3). WGS detected the toxin-associated gene *sta1* in four isolates. Of all analyzed isolates, the combination of the VAGs *fimH*, *papC* and *iucD* characterizing the UPEC pathotype was the most frequent one (23.52%), followed by the combination of a fimbrial gene/adhesion gene and a toxin gene characterizing the ETEC pathotype (22.54%). Further pathotypes were EDEC (4.90%), atypical ETEC and EPEC (each 3.92%), STEC (0.98%) and UPEC with enterotoxin (2.94%). In total, 40.19% of all *E. coli* isolates could not be assigned to a specific pathotype.

4. Discussion

This study aimed to characterize *E. coli* isolates from pig farms in Austria by using pheno- and genotyping methods as well as WGS. Resistance to antimicrobial agents was found in 81 (79%) isolates and 37 isolates met the MDR definition of Sweeney and colleagues [10]. Twenty-one isolates were susceptible to all antimicrobial agents tested. Resistance rates to penicillins (61.73%) and tetracyclines (58.81%) were similar to results of previous studies where penicillins and tetracyclines were the most common antibiotics with AMR in global pig production [42]. The distribution of resistance rates is similar to that in other European studies. Especially, an increased resistance to ampicillin was already reported in the EFSA surveillance program [43]. The variation in resistance in pathogenic *E. coli* was broad. This emphasizes the importance of performing antimicrobial susceptibility testing after pathotype identification for determining prognosis and guiding clinical management [44].

Colistin is considered by the WHO as a last-resort agent in the treatment of severe bacterial infections caused by multi-drug resistant Gram-negative bacteria [11]. Different genetic mechanisms are known to lead to colistin resistance. In particular, for isolates showing reduced susceptibility to colistin, this may be conferred by chromosomal alterations in *pmrAB* genes, which encode a two-component signal transduction system regulating the endogenous LPS modification system [45–47]. In 2015, the emergence and also the spread of mobile colistin resistance (*mcr*) genes were detected [48]. Although only three isolates in this study carried a MCR resistance gene, namely *mcr-1.1*, there is a scarcity of surveillance studies focusing on MCR genes in both human and veterinary medicine in Austria. Indeed, Austrian surveillance programs until now have not mentioned the presence of any colistin-resistant *E. coli* [49]. Only single reports from human medicine [50] and a study on the Austrian pig population reported the presence of MCR genes [50] previously. Regarding co-

resistance, the fact that two of the *mcr-1.1*-positive isolates showed MDR to penicillins, tetracyclines and trimethoprim–sulfamethoxazole highlights the threat of these clones to therapeutic choices [45]. In animal production, colistin is extensively used for metaphylactic and therapeutic purposes, which may contribute to increasing levels of colistin resistance [45]. For this reason, the European Medicine Agency has raised serious concerns in regard to the use of colistin in animals and the increasing risk for humans that this antimicrobial resistance poses [43].

In addition to colistin, fluoroquinolones are critically important antimicrobials and sometimes they are the sole or one of limited available therapies to treat serious bacterial infections in people (EARS Net Reports. Available online: https://www.ecdc.europa.eu, accessed on 29 July 2021). Resistance to fluoroquinolones among the investigated *E. coli* isolates was observed in 14/102 isolates (13.7%). Although results must be compared with caution because of the different methodologies performed, the proportion of samples with resistance to fluoroquinolones was lower than in other studies performed on humans, which was revealed to be 18.2% on average [51].

Different *E. coli* lineages are responsible for animal as well as for human *E. coli* infections, with previous studies having identified food and food animal reservoirs as sources for zoo-anthropogenic *E. coli* clones [52]. A study conducted on ESBL-positive *E. coli* isolates of human and animal origin in the Netherlands, the UK and Germany revealed that human *E. coli* isolates in the three countries were more closely related to one another than to isolates from animals [53]. In our study, we found isolates of distinct *E. coli* clonal lineages, including the specific international high-risk clone O25:H4-ST131-H22, which emphasizes its wide distribution and would be the first report of ST131 in pigs of Austrian origin. In addition, recent studies demonstrated the potential of *E. coli* O25:H4-ST131 to serve as a foodborne UPEC [54] and revealed the close relationship of human and porcine ST131 strains [55]. Indeed, enhanced virulence and antimicrobial resistance were compared with other *E. coli* ST131 strains from our recent work [56]. Interestingly, a number of virulence genes, encoding colonization, iron uptake, and biofilm formation, which are key enabling factors for the clinical success of ST131 [54,56–58], were present in both isolate types (24 VAGs in 24_8, 26 VAGs in 99_74).

Concerning *E. coli* ST10, an ancestral and ubiquitously occurring lineage comprising both commensal and pathogenic strains, it was detected in six out of 35 sequenced isolates. All but one isolate showed MDR, including a plasmid-predicted carriage of the *mcr-1.1* gene (IncX4) in two isolates and $bla_{\text{CTX-M-1}}$ (IncI1-I(Alpha)) in one isolate. Previous studies confirmed ST10 as the dominant ST from swine in Northern Europe with a broad host range and association with hospital- and community-acquired infections [59]. Shepard et al. [60] found that ST10 is one of the main *E. coli* clonal complexes associated with porcine ETEC, and Garcia et al. identified ST10 as primarily responsible for *mcr-4* spread [61]. Nevertheless, more investigations are necessary to verify if *E. coli* from porcine sources may be derived from the same bacterial lineages or share common evolutionary roots with human isolates.

The reporting of STEC O26 infections has been steadily increasing in the EU due to improved diagnostics of non-O157 sero-pathotypes (EARS Net Reports. Available online: https://www.ecdc.europa.eu, accessed on 29 July 2021). Among characterized *E. coli* strains, an atypical enteropathogenic *E. coli* (aEPEC), O26:H11_ST88, was detected. Besides the intimin (*eae*), which confers the ability to cause attaching and effacing (AE) lesions, the strain harbored heat-stable toxin gene *astA* and a further 20 VAGs. Previous studies described aEPEC as a possible progenitor of *stx*-producing O26:H11 STEC that is a major pathogen by causing severe gastrointestinal infections in animals and humans [62] and hemolytic–uremic syndrome (HUS) in humans [63]. Further studies indicated that aEPEC isolates may be able to acquire *stx* by integrating the s*tx*-prophage into their genome and further function as STEC [64]. In addition, the isolate in our study was MDR and harbored a plasmid-predicted *mcr-1.1* gene.

ETEC strains are recognized as the most common cause of porcine neonatal diarrhea (ND) and PWD in pigs [44], and were found in 23 of the investigated isolates. Interestingly, the pathotype UPEC was found to be the most common (24 isolates), although collected samples were mainly associated with ND and PWD. In total, 41 isolates could not be assigned to a specific pathotype because of lacking a specific combination of VAGs, or because of harboring VAGs that are specific for more than one pathotype. This circumstance may confirm expectations of Robins-Browne et al. and Müller et al. [3,65] that some of the typing schemes in current use will eventually be replaced, allowing more pathotypes to be identified (2016).

Phylogenetic analyses found groups A and B1 to be the most common, which corresponded to the results of similar studies [66]. Phylogroup B2 was represented by 4/102 isolates, all of which represented the UPEC pathotype, including both ST131 isolates, as previously confirmed by Nicolas-Chanoine et al. [67].

The plasmid types IncF, IncI and IncX, carrying VAGs and AMR genes, were found. These findings are a cause for concern, as these elements can easily be transferred from animal host pathogens to human pathogens, increasing their AMR and virulence [8]. IncF is the most frequently described plasmid type found in *E. coli* of human and animal sources. Interestingly, our investigation revealed that the *traT* gene, which codes for surface exclusion, was IncFI1-associated [68]. In a single isolate, $bla_{CTX\text{-}M\text{-}1}$ was predicted to be on an IncI1 plasmid. Such plasmids are predominantly described as $bla_{CTX\text{-}M\text{-}1}$ carriers in *E. coli* of European poultry and are further considered as a possible source for human infections [69]. In our study, three of 102 isolates carried the *mcr-1.1* gene on an IncX4 plasmid, which is in agreement with other works on Salmonella and *E. coli* isolates obtained from human and animal sources where IncX plasmids are also shown to carry *mcr* genes [69].

Biocides are applied as an integral part of infection control in pig production and slaughterhouses. The selection of bacteria with reduced susceptibility to disinfectants has already been confirmed [70]. In our study, we investigated biocide susceptibility and revealed unimodal MIC distributions for benzalkonium chloride, glutardialdehyde and isopropanol. In comparison, a bimodal MIC distribution was observed for chlorhexidine,

which might point towards the acquisition of the respective resistance properties. Previous studies confirmed that biocide-like disinfectants and surfactants are effective to select for AMR [71].

In our study, the newly developed oligonucleotide microarray offered an accurate and rapid solution to detect a large set of *E. coli* VAGs. Previous studies compared the accuracy and time needed to perform a microarray-based method with conventional multiplex PCR [72], and showed that microarray-based diagnostics was less labor-intensive and, therefore, more cost-effective. In addition, the error rates occurring in the amplification process during multiplex PCR do not exist when using microarrays [73]. Therefore, in our study, microarray technology offered an accurate and rapid tool to detect a large set of VAGs in parallel.

Conclusions

In this study, we have found porcine high-risk zoonotic *E. coli* clones that are both pathogenic and multi-drug resistant. The threat that these clones can pose to public health is derived from their AMR to critically important antibiotics for humans. Therefore, our work highlights the importance of monitoring AMR and VAGs in porcine *E. coli* isolates. This can be achieved by applying reliable, fast, economical, and easy to perform technologies such as DNA-based microarray typing. Nevertheless, preventive measures in swine farms in addition to surveillance must be applied to avoid infection of the pigs with resistant and pathogenic *E. coli* strains and to avoid their spread.

Limitations of Our Study

Data on prevalence, serotypes, and pathotypes of porcine *E. coli* in Austria and other countries were scarce, which made comparisons difficult. In our study, we were not able to compare our data on the national level because resistance in swine is not monitored yet in a harmonized way in Austria.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/microorganisms9081676/s1, Tables S1 and S2: Biocide susceptibility, Table S3: Plasmid presence_virulence genes_sorted, Table S4: Plasmid probability prediction—AMR genes, Table S5: Plasmid probability prediction_virulence genes, Table S6: Overview plasmid presence AMR genes.

Figure S1: Splitstree.

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A.L., J.S. and R.E.; data curation, A.C.-R., M.K., D.M., K.F., A.T.F., M.P.S., W.R., S.D.B. and I.L.; writing—original draft preparation, T.B.-H. and I.L.; writing—review and editing, A.C.-R., D.M., A.T.F., F.A., M.P.S., W.R., S.D.B., S.M., R.E., S.S. and

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