Research in Microbiology 167 (2016) 678–684

www.elsevier.com/locate/resmic

Original article

Genetic diversity, the virulence gene profile and antimicrobial resistance of clinical mastitis-associated *Escherichia coli*

Orla M. Keane

Animal & Bioscience Research Department, Teagasc, Grange, Dunsany, Co. Meath, Ireland

Received 24 March 2016; accepted 29 June 2016
Available online 9 July 2016

Abstract

Escherichia coli is a common cause of bovine mastitis, particularly around parturition and early lactation when the host is immunosuppressed. Isolates (n = 37) recovered from cases of clinical mastitis in Ireland were characterised with respect to genotypic diversity, phylogenetic group, virulence gene profile and antimicrobial susceptibility. The isolates were genotypically diverse, belonging to 19 different sequence types. However, the majority (86%) belonged to phylogenetic groups A or B1, groups commonly associated with commensal *E. coli*. The isolates encoded few virulence genes with *iss* (increased serum survival, 41%), *lpfA* (long polar fimbriae, 19%) and *astA* (enteroaggregative heat-stable toxin, 14%) among the most common virulence genes detected. The only virulence gene to differ in frequency between the phylogenetic groups was *lpfA*, found exclusively in B1. Resistance to at least one antimicrobial was detected in 16% of isolates. Three isolates were multidrug-resistant, with one resistant to seven antibiotics. There was no relationship between antimicrobial resistance and phylogenetic group. These results indicate that many cases of clinical *E. coli* mastitis in Ireland may be caused by opportunistic commensal organisms lacking specific virulence genes. However, the organisms represent a reservoir of antimicrobial resistance determinants with the potential to disseminate determinants to other organisms.

Keywords: Clinical mastitis; Pathogen; *E. coli*; Virulence gene; MLST

1. Introduction

Intramammary infection (IMI) is a common disease in the dairy industry internationally, with considerable economic costs [1,2]. IMI has a negative impact on animal health and welfare, animal productivity and on public perception of the quality of dairy products. IMI is also the main cause of antimicrobial use in the dairy industry [3] which is a public health concern due to the potential for the emergence and spread of antibiotic-resistant bacteria or the transfer of antibiotic resistance genes to human pathogens [4–6].

A common cause of bovine clinical IMI is *Escherichia coli* [7–9]. *E. coli* has traditionally been considered an environmental pathogen in its mode of transmission and to opportunistically enter the udder via the teat canal. *E. coli* infections commonly occur during parturition or in early lactation when the host immune response is suppressed [10], and can result in severe or acute clinical mastitis with a high bacterial count and a large influx of host somatic cells [11]. However, infections are often of short duration with rapid clearance of bacteria and a drop in somatic cells over a short period of time.

Distinct clonal groups of *E. coli* have been associated with a variety of human and animal diseases [12–14], suggesting that these clones are pathoadapted to cause a particular disease. Phylogenetic analyses have shown that the majority of *E. coli* isolates belong to one of four major phylogenetic groups (A, B1, B2 or D), with many commensal isolates belonging to group A, while group B2 and, to a lesser extent, group D, have been associated with extra-intestinal disease [15]. It has also been reported that genetically distinct strains of *E. coli* exist which may have adapted to the bovine intramammary milieu [16] and

E-mail address: orla.keane@teagasc.ie.

http://dx.doi.org/10.1016/j.resmic.2016.06.011

can give rise to persistent infections [17]. The aim of this study was to determine the genetic diversity of *E. coli* strains causing bovine clinical mastitis in Ireland by determining their clonal relatedness and virulence gene profile. The antimicrobial resistance status of the isolates was also investigated.

2. Materials and methods

2.1. E. coli *strain isolation*

A total of 53 *E. coli* isolates were recovered from milk samples from cows presenting clinical mastitis between February 2010 and February 2011 in Ireland. Sample collection, bacterial isolation and pathogen identification methods have been described previously [7]. Only isolates where *E. coli* was the only species recovered from the infected mammary gland were retained to avoid the inclusion of potential environmental contaminants, resulting in 37 isolates for study. The isolates were from 18 different farms located in 5 counties and were from different animals, with the exception of 2 isolates which came from the same animal but from different quarters, and were isolated 56 days apart and so were considered separate infections. Glycerol-stored (−80 °C) cultures were subcultured on Luria–Bertani (LB) agar and used for further study.

2.2. *Phylogenetic grouping*

Assignment to one of the ECOR phylogenetic groups (A, B1, B2 or D) was performed by PCR using the primers described in Ref. [15]. DNA was extracted from each isolate using the PurElute Bacterial Genomic kit (EdgeBio) and tested for the presence of *chuA*, *yjaA* and TspE4.C2. Reactions were carried out in 1× Phusion HF buffer, 0.2 mM each dNTP, 0.5 μM of each primer, 1 U Phusion (New England Biolabs) and approximately 100 ng genomic DNA. The reactions were denatured at 98 °C for 30 s and then cycled 35 times at 98 °C for 10 s, 68 °C for 15 s and 72 °C for 30 s. Reactions were then extended at 72 °C for 5 min.

2.3. *Multilocus sequence typing*

MLST profiles were analysed following the method of Ref. [18] with some minor modifications as described below. Loci *adk*, *fumC*, *gyrB*, *icd*, *mdh* and *recA* were amplified with the previously described primers [18]. For the *purA* locus, amplification primers purA1F 5′ TTCTCCGCGAGAATGTAACC 3′ and purA1R 5′ CCATCCAGAACGTCCAGTTT 3′ were used. The PCR reactions were carried out as described above. The reactions were denatured at 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 60–66 °C for 10 s and 72 °C for 30 s. Reactions were then extended at 72 °C for 5 min. The following annealing temperatures were used; *icd* at 60 °C, *adk*, *fumC* and *purA* at 62 °C, *recA* at 64 °C and *gyrB* and *mdh* at 66 °C. The products were sent to Source Bioscience (Dublin, Ireland) or Beckman Coulter Genomics (Takeley, UK) for Sanger sequencing. All products were sequenced with both amplification primers. The sequence chromatograms were checked for quality using BioEdit v 7.0.0 and allelic profiles were assigned by submitting to the *E. coli* MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli).

2.4. *Microarray and* tetA *genotyping*

Sensitive and specific miniaturised microarrays encoding gene targets for the identification of *E. coli* virulence genes, antimicrobial resistance genes and DNA-based serotyping were used for genetic characterisation of the *E. coli* isolates. The details of the arrays have been reported previously [19–21] and a complete list of targets is available at http://alere-technologies.com/fileadmin/Media/Downloads/op/10313/13-11-19_0004_V01_User_guide_E.coli_combined_assay.pdf. Isolates classified as ambiguous were treated as negative for analysis purposes. The *tetA* gene was amplified with primers tetAF 5′ GTAATTCTGAGCACTGTCGC 3′ and tetAR 5′ CTGCCTGGACAACATTGCTT 3′ [22] as described above, with an annealing temperature of 64 °C.

2.5. *Antimicrobial resistance*

Antimicrobial susceptibility testing was performed for all *E. coli* isolates by the disk diffusion method in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines [23]. Ampicillin (10 μg), amoxicillin and clavulanic acid (20 μg + 10 μg), chloramphenicol (30 μg), cefalexin (30 μg), ceftiofur (30 μg), enrofloxacin (5 μg), neomycin (30 IU), kanamycin (30 μg), streptomycin (10 μg), tetracycline (30 μg) and trimethoprim and sulfamethoxazole (1.25 μg + 23.75 μg) were tested. The *Staphylococcus aureus* American Type Tissue Collection (ATCC) 25923 and *E coli* ATCC 25922 strains were used as quality control standards and isolates were classified as resistant, intermediate or susceptible based on comparison of the measured zone diameter with the zone diameter interpretive standards for veterinary pathogens. Isolates classified as intermediate were considered susceptible for analysis purposes. There are currently no CLSI breakpoints approved to indicate ceftiofur or neomycin resistance in Enterobacteriaceae, and so the veterinary interpretative criteria for cattle respiratory disease was used in the case of ceftiofur [24] and a resistant zone diameter breakpoint of ≤12 mm for neomycin [25].

2.6. *Phylogenetic and statistical analysis*

Population-scaled mutation and recombination parameters were estimated using ClonalFrame v1.1 [26]. The concatenated sequence of each of the seven MLST loci was used as input and a single representative of each sequence type was used in order to minimise bias resulting from over-representation of particular clones. Five independent runs of the Markov chain were performed with 100,000 burn-in iterations and a posterior sampling of 200,000 iterations. The burn-in iterations were discarded and model parameters were estimated every 100 iterations from the posterior. Satisfactory