Evaluation of the Virulence Factor Profile of Avian Pathogenic *Escherichia Coli* in Clinical Isolates of Avian Samples in Caloto, Cauca, Colombia

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Abstract

Avian pathogenic *E. coli* (APEC), produces an extraintestinal infection in chickens, turkeys, and other types of birds, called colibacillosis, and is considered one of the main causes of economic losses due to morbidity, mortality, and the disposal of poultry carcasses. The objective of the present study was to characterize the genetic profile of the virulence factors of different isolates of avian *E. coli* in Caloto, Cauca, Colombia.

Materials and methods: *E. coli* was isolated and identified by biochemical tests, from 47 clinical isolates. Subsequently, the DNA was extracted using Chelex. Three multiplex PCRs were designed to amplify 13 virulence factors (*iroN, hlyF, iss, iutA, frz, vat, sitA, KpsM, sitD, fimH, pstB, sopB, and uvrY*), using primers previously reported for each. The amplification products were verified on agarose gels. Each isolate was classified according to the number of virulence factors: group A (between 10 and 13), group B (between 5 and 9), and group C (4 or less). Conclusion: we were able to identify the presence of a group of virulence factors in clinical isolates of APEC, which allows us to demonstrate that both the frequency and the profile of virulence factors in the isolated strains showed a different profile than the reported by other authors. The virulence genes *pstB* and *fimH* were detected in all of our samples, and the *iss* gene was the one with the lowest frequency. And according to the number of virulence factors, the group A was the most frequent.

Introduction

Escherichia coli is a gram-negative bacillus that belongs to the *Enterobacteriaceae* family and is considered to be a member of the extraintestinal pathogenic *E. coli* (ExPEC) group. Some strains are potentially pathogenic and can cause serious diseases in humans and animals, such as urinary tract infection, neonatal meningitis, septicemia, and even in some cases can be fatal[1,2]. Avian pathogenic *E. coli* (APEC), is among the strains that produce an extraintestinal infection in chickens, turkeys, and other types of birds, called colibacillosis[2], characterized by an initial respiratory disease, followed by a systemic infection that induce fibrinous lesions in different organs, causing airsacculitis and associated pericarditis, perihepatitis, as well as, cellulitis, peritonitis, and fatal septicemia[3,4]. APEC is considered one of the main causes of economic losses due to morbidity, mortality that in some cases can even reach almost 20%, and disposal of poultry carcasses worldwide [5–7].

The *E. coli* strains responsible for these extraintestinal infections in birds have several genes that encode virulence factors related to processes such as adhesion, invasion, iron acquisition systems, hemolysis, immune response evasion, resistance to antibiotics, and toxin production[7,8]. These virulence genes are encoded in the genome of *E. coli* or plasmids[9], they are not present in all isolates, in some cases, one or multiple virulence genes can occur, and some of these have been detected in isolates from healthy birds. Although it has been reported that the pathogenicity of APEC strains is likely to be determined by the presence of at least five virulence genes[3,8,10], the specific genes conferring to APEC virulence are less well described than in human ExPEC pathotypes, in consequence, has been difficult to understand whether it is just one or a combination of virulence factors associated with the strains what causes the disease[5].

In the present study, the evaluation of 13 virulence factors of APEC (*iroN, hlyF, iss, iutA, frz, vat, sitA, KpsM, sitD, fimH, pstB, sopB, and uvrY*) was performed, they were selected thanks to a review of the virulence genes that have been most frequently reported in APEC isolates in other countries[9,11–13]. The salmochelin siderophore receptor gene, *iroN*, facilitates the chelation of iron in the host[14]. A putative avian hemolysin gene, *hlyF* and an aerobactinsiderophore receptor gene, *iutA*, both contribute to iron absorption[15]; as well as, the episomic increase of the serum survival gene, *iss*, which helps with resistance to host serum[16]. The *frz* operon promotes bacterial fitness under stressful conditions[17]. The *vat* gene encodes a vacuolating autotransporter toxin and it has been reported that could be related to agglutination, biofilm formation as well as virulence[18]. The *sitA* and *sitD* genes are part of the sitABCD system, classified as a bacterial iron transporter[19]. The capsule formation transporter gene, *KpsM*, encodes a polysaccharide protein transporter for the formation of protective

capsule[20]. The type 1 fimbrial adhesion gene, *fimH*, contributes to the protection from the host heterophile antibodies[21]. The *pstB* gene which is part of the pstSCAB operon, has been shown to increase resistance to polymyxin, rabbit serum and acid shock[22]. The *sopB*-encoded plasmid division protein is common in several plasmids, including pAPEC-1 (GenBank accession number CP000836), pAPEC-O1-ColBM (GenBank accession number DQ381420) and pVM01 (GenBank accession number EU330199) associated with virulence traits in APEC[19]. And a transcriptional regulatory gene of iron uptake genes in APEC, *uvrY*, which is involved in the regulation of carbon metabolism and contributes to its virulence[23].

Antibiotics have been for a long time the first line of defense to prevent APEC, but they have lost their clinical efficacy as bacteria have become increasingly resistant to treatment due to their irrational use, and at the moment there is no effective vaccine because of the multitude of serotypes involved[24,25]. Accordingly, it has been reported a high prevalence of multidrug-resistant strains among causal isolates of colibacillosis [26,27], as well as, outbreak alerts in recent years where multi-resistant APEC has been identified[28,29]. It seems that these strains are not only facilitating the transmission and dissemination of drug resistance and other virulence factors between animal and human pathogens, but also they could enhance antimicrobial resistance in other organisms (pathogenic and nonpathogenic) within gastrointestinal tract of the chicken[30,31]. In addition, APEC share not only identical serotypes with human pathogens but also specific virulence factors, therefore their zoonotic potential is under consideration and is highly concerning[32–35].

Genetic diversity is an obstacle for the identification of common properties, which could be used as a basis for diagnostic methods and vaccination, this challenging condition is related to the arduous control of avian colibacillosis. Due to this, the identification and characterization of virulence genes have emerged as a need to develop specific therapeutic targets that could contribute to the implementation of new strategies for treatment.

In Colombia, to date, exists some reports related to antibiotic resistance which in this case has an intricate relation to virulence, however, no studies have been conducted evaluating the presence of virulence factors in isolated strains of APEC, which makes it difficult to determine specific strains for vaccine development, the effectiveness of antibiotics or even the prevalence among infections in the community. The present work aimed to characterize the genetic profile of some virulence factors of different isolates of avian *E. coli* in Caloto, Cauca, Colombia.

Results

A total of 47 *E. coli* strains were isolated from the cultures of oviduct, lung, and liver samples. A bacterial pool containing *E. coli* strains that had different virulence factors was used as a positive control since no strain possessed all the virulence factors studied. The three multiplex PCRs were run with the DNA pool to ensure that under the proposed conditions the different genes were amplified (Fig. 1).

The selected genes were amplified from the pool of control strains, and in the different isolates. For the multiplex PCR 1, a total of 37 isolates (78.7%) were positive for *iroN*, and 10 (21.3%) were negative, 38 isolates (80.9%) were positive for *hlyF*, and 9 isolates (19.1%) were negative, 3 isolates (6.4%) were positive for *iss*, and 44 (83.6%) were negative, and 36 isolates (76.6%) were positive for *iutA*, and 11 (23.4%) were negative. The profile of the bands generated can be seen in Fig. 2.

In the multiplex PCR 2, a total of 23 isolates (48.9%) were positive for *frz*, and 24 (51.1%) were negative, 30 isolates (63.8%) were positive for *vat*, and 17 (36.2%) were negative, 35 isolates (74.5%) were positive for *sitA*, and 12 (25.5%) were negative, and 19 isolates (40.4%) were positive for *KpsM*, and 28 (59.6%) were negative. The profile of the bands generated can be seen in Fig. 3.

In multiplex PCR 3, a total of 32 isolates (68.1%) were positive for *sitD*, and 15 (31.9%) were negative, all 47 isolates (100%) were positive for *fimH*, as well as for *pstB*, 32 isolates (68.1%) were positive for *sopB*, and 15 (31.9%) were

negative, and finally 11 isolates (23.4%) were positive for *uvrY*, and 36 (76.5%) were negative. The profile of the bands generated can be seen in Fig. 4.

The isolates were classified into three profiles according to the virulence factors detected. In profile A, the isolates that had between 10 and 13 virulence factors. In profile B, those that possessed between 5 and 9 virulence factors, and finally in profile C, those who had 4 or less virulence factors, which based on the genetic criteria for the pathogenicity were considered as the avian nonpathogenic Escherichia coli (non-APEC) strains[3,10]. Out of 47 *E. coli* isolates, 39 (82.9%) isolates were found to be likely APEC strains due to the number of virulence factors, and 8 (17.02%) isolates were found to be non-APEC strains. For profile A, 20 isolates were found, and the maximum number of virulence factors detected was 12. For profile B, 19 isolates, and for profile C, 8 isolates, and the lowest number of virulence factors detected was 2. Among 47 *E. coli* strains, 1 strain contained twelve of the thirteen virulence genes evaluated, 13 strains contained eleven virulence genes, 6 strains contained ten virulence genes, 9 strains contained nine virulence genes, 5 strains contained eight virulence genes, 4 strains contained seven virulence genes, 1 strain contained six virulence genes, 1 strain contained 4 virulence genes, 2 strains contained 3 virulence genes, and 5 strains contained 2 virulence genes. The profile of the virulence factors of the vaccine strain was classified within profile C, with only 4 virulence factors (Table 2).

Discussion

It has been calculated that there are over 26 billion chickens worldwide, with poultry constituting around 70% of all bird biomass on earth[5,36]. According to the National Federation of Poultry Farmers (FENAVI) in Colombia, the poultry industry has grown considerably in the past decade, providing an annual output of more than one million tons of chicken meat during 2018, due to the implementation of hazard analysis and critical control point (HACCP) programs, in slaughter poultry establishments as a voluntary measure to improve food safety. And even though Colombian farms meet international standards of environmental and animal welfare, which allow them to serve markets with special requirements[37,38], the accurate data of prevalence of multi-antibiotic resistant strains or even pathogenic strains in Colombia is hardly documented, or at least reported, with exception of some initiatives.

The Colombian Integrated Surveillance Program for Antimicrobial Resistance (COI-PARS) was established as a pilot project to monitor antimicrobial resistance among bacteria on poultry farms, slaughterhouses, and retail markets. This project helped to validate the methodology in the poultry chain in Colombia[39]. In 2015 as a continuance of COIPARS, Donado-Godoy and collaborators established the baseline antimicrobial resistance patterns of *Salmonella* serovars, *Escherichia coli*, and *Enterococcus* spp., noticing that almost 98% of isolates tested were multidrug-resistant[39]. As a complement, Ramírez-Hernández and collaborators provided reference data for *E. coli* levels at various chicken processing steps in slaughterhouses in three representative regions of Colombia[38]. Finally, during 2017 Castellanos et al. reported that the resistance to extended-spectrum cephalosporins in *E. coli* from Colombian poultry samples, was mainly caused by Extended Spectrum Beta-Lactamases (ESBLs), and AmpC beta-lactamases, ESBL/AmpC genes including bla_{CMY-2} and bla_{SHV-12} [40].

Because of the lack of data and information related to APEC in our country, and regardless that some evidence suggests that most natural microflora related to poultry production is not pathogenic to humans[41,42], it is early to know if APEC and ExPEC strains are phylogenetically related sharing some of the same virulence genes, and if there is a connection to antibiotics resistance, especially in APEC phylotypes which has been explored in other countries[8,43,44]. All of this represents not only an important human, and animal health threat that requires persistent public health vigilance, but also a menace to the food chain production, as it has been previously reported for some studies[5,45–47].

In this sense, the results of the three different multiplex PCRs have allowed us to detect a large number of virulence factors in our *E. coli* isolates, which have shown a high prevalence in previous studies. The *E. coli* isolates in this study

had at least two, and a maximum of 12 virulence genes, although the pathogenicity of APEC strains can be determined by the presence of at least five virulence genes[3,10,48–50]. And the frequency of each virulence factor when compared to previous reports worldwide, contributes to making a differentiation concerning the frequency of each factor in our isolates.

In the case of *iroN*, frequencies between 56 and 100% in APEC isolations have been reported[15,51–53]. In our isolates was closer to 80%, and as part of ExPEC strains, some studies had provided evidence that this iron uptake system may also contribute to adherence and invasion during infection[54]. Our study showed that the frequency of the *iroN* gene was closer to that found in the United States, Canada, and Nepal, than to those conducted in South Africa and Sir Lanka.

The *hlyF* gene is epidemiologically associated with virulent strains in APEC and also human neonatal meningitis–associated *E. coli*. Recent studies have reported frequencies of 93.7% in Korea and 100% in Nepal. From the study in Nepal, they also managed to demonstrate within *E. coli* strains, a correlation between the presence of virulence factors and antibiotic resistance[55,56]. In Brazil, the authors were able to detect the presence of this gene in 71.4% of the isolates[57]. Our frequency was 80.9%, which showed to be higher than those reported in other South American countries, but lower than the reported in Asia. And that its absence is in those isolates with seven or fewer virulence factors, which might be related to less virulent strains. This virulence factor is directly involved in the production of outer membrane vesicles, and their increased production was associated with the release of toxins during extraintestinal infection[58].

Regarding the *iutA* gene, the reported frequencies were very variable among the isolates, between 36%, and 100%[10,28,53,59]. In our study, the frequency was 76.6%, similar to *iroN* gene frequency (78.7%), both of these virulence genes encode siderophores and are essential for self-maintenance in the host body in APEC, and human ExPEC strains, contributing to their virulence[59]. On the other hand, the *Iss* gene constantly has been reported in several studies with higher frequencies between 80 and 100% in the United States, Spain, Denmark, Nepal, Qatar, Korea, Canada, and Jordan[10,29,53,60–64]. Another study from the University of Florida reports a frequency of 75%, in which they also established that the presence of this gene could be associated with a health risk for humans[65] And even lower frequencies have been reported in isolates from Brazil (38.5%, 39.2%, and 49.5%)[57,66,67]. However, our frequency was 6.4%, showing a huge difference from what was already reported.

The *frz* gene frequency has been reported in a study conducted in France with 15.1% [7], and 22% in South Africa[15]. However, the results of this study agree with those from Schouler *et al* .with samples from France, Spain, and Belgium from chickens, turkeys, and ducks exhibiting clinical symptoms of different forms of colibacillosis, in which they found a prevalence of 53.4% compared to ours with 48.9%[7]. In the case of the *fimH* gene, as it has been reported in Brazil, the United States, South Africa, and our study in Colombia, the frequency can vary from more than 80–100%, in our case *fimH* was detected in all the samples[15,57,65]. Related to the expression of these genes, the gene *frz* is chromosomally located and belongs to the *frz* operon, which encodes a phosphoenolpyruvate carbohydrate phosphotransferase system transporter and enzymes involved in sugar metabolism, which has been proven relevant not only promoting bacterial fitness under stressful conditions but also seems to be involved in the cell surface expression of F1 fimbriae[17].

The *vat* and *sitA* genes have been identified in both APEC and uropathogenic *E. coli* (UPEC) strains, both of them are present in more than 60% of our strains. It has been demonstrated with *in vitro*, and *ex vivo* models that vacuolating autotransporter toxin induced cellular damage, vacuole formation, and urothelial barrier dysregulation of bladder epithelial cells[68]. The frequency of the *vat* gene in Brazil was 13.2%[57], while in Nepal reach out 89%[10], and in England around 11.25%[48]. In our isolates, the frequency was 63.8%, much higher than the one found in Brazil and England, but lower than in Nepal. For the *sitA* gene was reported a 20% in England[48], and with very similar values in Canada with 20.57%[69]. However, *sitA* was also found in over 85% of APEC, and as well human UPEC isolates, suggesting according to the authors, that it might enable APEC strains to cause extraintestinal disease in human beings[13,70]. In recent studies of APEC

isolates the most common gene identified was *sitA*, one from broiler and broiler breeder chickens in Ontario, Canada detected in 93% of the isolates[53] and another in broiler chickens in Jordan, with 97.4%[61], our study shows a frequency of 74.5%.

Our results showed that the gene frequency of *KpsM*, *sitD*, and *pstB* are higher than reported. The *kpsM*, *pstB* genes had the lowest prevalence, with only 2.2% in Zimbabwe[71]. In the case of the *KpsM* gene, a frequency of 10% in the United States[65], in Brazil of 14.3%[57], and our isolates in Colombia was around 40%. The frequency of the *sitD* gene was 11% in Brazil, 50% in Canada[69], and 42.8% in South Africa[15]. We reported a frequency of 68.1% in our isolates. And for the *pstB* gene, almost 86% in South Africa[15], and in our isolates, the frequency was 100%.

It has been reported that the *sop*B and *uvrY* genes have not been detected in different avian fecal *E. coli* (AFEC) isolates from a variety of species of birds, such as turkeys, geese, and ducks[72]. However, the gene *sopB* is not only highly prevalent among the Salmonella pathogenicity island genes and has a relevant impact on the pathogenesis and epidemiology of Salmonella infections in poultry[73–75], but also was detected among the most prevalent virulence associated genes in APEC isolates, from confirmed cases of colibacillosis in chickens in Bulawayo, Zimbabwe, with a 20%, contrasting a 4.4% for the *uvrY* gene[71]. In addition, from 10 Zimbabwean APEC isolates, the gene *uvrY* was detected in all the samples and the *sopB* gene in the 30%[15]. In our isolates, the *sopB* gene was detected in 68.1% and the gene *uvrY* in 23.4% of them.

We were able to identify the presence of a group of virulence factors in bird clinical isolates of APEC in the Caloto region in Colombia, that share similarities as well as differences in both the frequency and the profile of virulence factors of those reported by other authors in other countries. As a particular trait *pstB* and *fimH* genes were present in the total of our isolates, and *Iss* gene had the lowest frequency, and the most common profile, was profile A, strains with between 10 and 13 factors of virulence.

According to published reports, the ColV plasmid has been considered an epidemiological marker of APEC, our study reported the presence of four of the five ColV plasmid-encoded genes (*IroN, hlyF, iss*, and *iutA*), more than 80% of our strains showed one or a combination of at least 3 of these genes. In the case of *iss* despite the fact is considered as a candidate target of colibacillosis control procedures, and is strongly associated with APEC strains, it has been also detected in AFEC isolates from healthy birds[66,76–78]. Our results showed a very low frequency, that could be related to varying copy number of *iss* gene and hence its detection in our strains, or maybe related to individual genetic characteristics of the animals[79]. Likewise, it has been shown that some *E. coli* strains isolated from colibacillosis cases, lacks important virulence genes without losing their pathotype and that there is no knowledge of a specific gene to be essential for the development of the extraintestinal infection in birds contributing to APEC virulence, and hence their relation with human infection. According to this, and following the concept that pathogenicity is multifactorial and can arise from enriched commensal populations[5], we are not only should be aware of the relationship between the presence of genes and *E. coli* virulence but also the effect of the sequence and transcription level, as well as the role of the intensity of the disease, risk factors, even environmental conditions[67,80], potential antimicrobial resistance, and the frequency of virulence genes from isolates of healthy chickens[66]. Which in our country, through more defined and effective molecular monitoring could end up in improved animal welfare and human food chain safety.

Methods

Sample collection

A total of 47 *E. coli* strains were isolated from the cultures of oviduct, lung, liver, coelomic cavity and yolk sac samples taken from animals, diagnosed with bursitis in the area of Caloto, Cauca, Colombia.

Isolation and bacterial identification

The bacteria were isolated on MacConkey agar, subsequently, they were identified using biochemical tests, and finally, 3 isolated colonies of each strain were placed in sterile PBS for DNA extraction.

DNA extraction

The DNA was extracted using Chelex® 100. For this method, 100 µl of the culture was centrifuged at 12,000 rpm for 5 minutes, then the supernatant was removed and the pellet resuspended in 100 µL of miliQ water, then vortexed for 30 seconds and added 100 µL of Chelex 10%. The samples were incubated at 99°C for 20 minutes in a Vortex at 70 rpm, left at room temperature for 10 minutes, and then centrifuged at 12,000 rpm for 10 minutes. Finally, the supernatant was transferred to a new 1.5 ml tube and stored at -20°C until use.

The amount of DNA in each of the extractions was determined using a Nanodrop (ND1000, Thermo Scientific, Wilmington, USA). For the evaluation of the integrity of the DNA, electrophoresis was performed using 1% agarose gel, containing 0.5 µg/ml of ethidium bromide. Images were acquired using photodocument of Chemidoc gels (Biorad) and the quantityOne software.

Oligonucleotide design

The genes selected to amplify in this study were chosen according to literature reports as previously mentioned. For each of the virulence factors previously reported primers were used, the size of the amplified fragments was between 198 bp and 843 bp (Table 1).

The PCR reaction mixtures were subjected to the following conditions in a Biorad 1000 thermocycler (Biorad, CA, USA): denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 63°C for 30 sec and 72°C for 3 min and a final cycle of 10 min at 72°C. The reaction mixture contained a final volume of 25 µl, thus: 2 µl of DNA, 2.5 µl of the buffer, 0.25 mM of dNTP (1.2 µl), 3 mM MgCl2 (1.5 µl), oligonucleotides 0.25-1 µM (0.625 µl) Taq polymerase 0.2 µl and nuclease-free H2O 13,925 µl. The products were visualized by electrophoresis using 3% agarose gel (w/v) in a 1X TAE buffer run at 100 V for 80 minutes.

Analysis of results

The results were analyzed to generate the following virulence profiles according to the number of genes related to virulence factors found in the different strains: profile A, isolates that had between 10 and 13; profile B, those that had between 5 and 9 and in profile C, those who had 4 or less virulence factors.

Declarations

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Contributions

H.D. Designed the study, revised the manuscript. H.M. Designed the study, revised the manuscript. E.M. Designed the study, processed the samples, analysed the data and wrote the manuscript. L.E. analysed the data and revised the manuscript. N.Q analysed the data and wrote the manuscript. R.V. Designed the study, processed the samples, analysed the data and wrote the manuscript.

Ethics declarations

The authors declare that they have no competing interests.

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Tables

Table 1

List of primers for each gene, sequence, size of each fragment to be amplified, concentration of the primer used for each multiplex PCR and reference from which the sequences were taken.

Gene Multiplex 1	Sequence 5´-3´	Size Fragment	Concentration µM	Reference
iroN F	AATCCGGCAAAGAGACGAACCGCCT	553 pb	0.25	(Johnson et al., 2008)
iroN R	GTTCGGGCAACCCCTGCTTTGACTTT		0.25	
hlyF F	GGCCACAGTCGTTTAGGGTGCTTACC	450 pb	0.25	(Johnson et al., 2008)
hlyF R	GGCGGTTTAGGCATTCCGATACTCAG		0.25	
Iss F	CAGCAACCCGAACCACTTGATG	323 pb	0.25	(Johnson et al., 2008)
Iss R	AGCATTGCCAGAGCGGCAGAA		0.25	
iutA F	GGCTGGACATCATGGGAACTGG	302 pb	0.25	(Johnson et al., 2008)
iutA R	CGTCGGGAACGGGTAGAATCG		0.25	
Gene Multiplex 2				
frz-FP	GAGTCCTGGCTTGCGCCGTT	843 pb	0.5	(Van der Westhuizen and Bragg, 2012)
frz-RP	CCGCTCCATCGCAGCCTGAA		0.5	
vat-FP	CGCTTCAGGTGCGCTGACCA	498 pb	0.5	(Van der Westhuizen and Bragg, 2012)
vat-RP	AAGGGAGACGATGCCCGCCT		0.5	
sitA-F	CGCAGGGGGCACAACTGAT	661 pb	0.5	(Sabri et al., 2008)
sitA-R	CCCTGTACCAGCGTACTGG		0.5	
KpsM-FP	CAGCCTCGCGGCTTAGCTCC	335 pb	0.5	(Van der Westhuizen and Bragg, 2012)
KpsM-RP	TGCACGCGCACTGCTTGAGA		0.5	
Gene Multiplex 3				
sitD-F	CTGTGCGCTGCTGTCGGTC	571 pb	0.6	(Sabri et al., 2008)
sitD-R	GCGTTGTGTCAGGAGTAC		0.6	
fimH-FP	GGATAAGCCGTGGCCGGTGG	331 pb	0.6	(Van der Westhuizen and Bragg, 2012)
fimH-RP	CTGCGGTTGTGCCGGAGAGG		0.6	
pstB-FP	CGCGCTCGTCCATGTCAGCA	198 pb	0.6	(Van der Westhuizen and Bragg, 2012)
pstB-RP	CGGAACAGCGTGCGGAAGGT		0.6	
*sop*B-FP	ACGACCCCCAGGGAACAGCA	797 pb	1	(Van der Westhuizen and Bragg, 2012)
*sop*B-RP	TGCAGCTGGTGCCGATGACG		1	
uvrY-FP	TGAGTGCGATTCGTTCTGTC	286 pb	0.6	(Herren et al., 2006)
uvrY-RP	TCTCCGCATTACACAGACCA		0.6	

Table 2
List of analyzed samples, PCR result for each virulence factor [negative (-) and positive (+)] and virulence profile.

Sample	Virulence Factors													Virulence Profile
	iroN	hlyF	iss	iutA	frz	vat	sitA	KpsM	sitD	fimH	pstB	sopB	uvrY	
1	+	+	-	+	-	+	+	-	-	+	+	-	-	B
2	+	+	-	+	+	+	+	+	-	+	+	-	-	B
3	+	+	-	+	-	+	+	+	-	+	+	-	-	B
4	+	+	-	+	-	+	+	+	-	+	+	-	-	B
5	+	+	-	+	-	+	+	+	-	+	+	-	-	B
6	+	+	-	+	-	+	+	+	+	+	+	+	-	A
7	+	+	-	+	-	+	+	+	+	+	+	-	-	B
8	-	-	-	-	-	+	-	+	+	+	+	+	-	B
9	+	+	-	+	+	+	+	-	-	+	+	-	-	B
10	+	+	-	+	+	+	+	+	+	+	+	+	-	A
11	+	+	-	+	-	-	-	-	+	+	+	+	-	B
12	+	+	-	+	-	-	-	-	+	+	+	+	-	B
13	+	+	-	+	-	+	+	+	+	+	+	+	-	A
14	+	+	-	+	+	+	+	-	+	+	+	+	-	A
15	+	+	-	+	+	+	+	+	-	+	+	-	-	B
16	-	+	-	+	-	-	-	-	-	+	+	-	-	C
17	+	+	-	+	+	+	+	+	+	+	+	+	-	A
18	+	+	-	+	+	+	+	+*	+	+	+	+	+	A
19	+	+	-	+	+	+	+	+	+	+	+	+	-	A
20	+	+	-	+	+	+	+	+	+	+	+	+	-	A
21	+	+	-	+	+	+	+	+	+	+	+	+	+	A
22	+	+	-	+	-	+	-	-	+	+	+	+	+	B
23	+	+	-	+	+	+	+	-	+	+	+	+	-	A
24	+	+	-	+	+	+	+	+*	+	+	+	+	+	A
25	+	+	-	+	+	+	+	+*	+	+	+	+	-	A
26	+	+	-	+	+	+	+	+	+	+	+	+	-	A
27	+	+	-	+	+	+	+	+	+	+	+	+	-	A
28	+	+	-	+	+	+	+	+	+	+	+	+	-	A
29	-	-	-	-	-	-	-	-	-	+	+	+	-	C

Sample	Virulence Factors													Virulence Profile
	iroN	hlyF	iss	iutA	frz	vat	sitA	KpsM	sitD	fimH	pstB	sopB	uvrY	
30	-	-	-	-	-	-	-	-	-	+	+	+	-	C
31	-	-	-	-	-	-	-	-	-	+	+	-	-	C
32	-	-	-	-	-	-	-	-	-	+	+	-	-	C
33	-	-	-	-	-	-	-	-	-	+	+	-	-	C
34	-	-	-	-	-	-	-	-	-	+	+	-	-	C
35	-	-	-	-	-	-	-	-	-	+	+	-	-	C
36	+	+	-	+	+	+	+	-	+	+	+	+	+	A
37	+	+	-	+	-	-	+	-	+	+	+	+	-	B
38	+	+	-	+	+	-	+	-	+	+	+	+	-	B
39	+	+	+	-	-	-	+	-	+	+	+	+	+	B
40	-	-	+	-	-	-	+	+	+	+	+	-	+	B
41	+	+	-	+	+	+	+	-	+	+	+	+	+	A
42	+	+	+	-	-	-	+	-	+	+	+	+	+	B
43	+	+	-	+	+	+	+	+	+	+	+	+	-	A
44	+	+	-	+	+	-	+	-	+	+	+	+	-	B
45	+	+	-	+	-	+	+	-	+	+	+	+	+	A
46	+	+	-	+	+	+	+	-	+	+	+	+	+	A
47	+	+	-	+	+	-	+	-	+	+	+	+	-	B

Figures

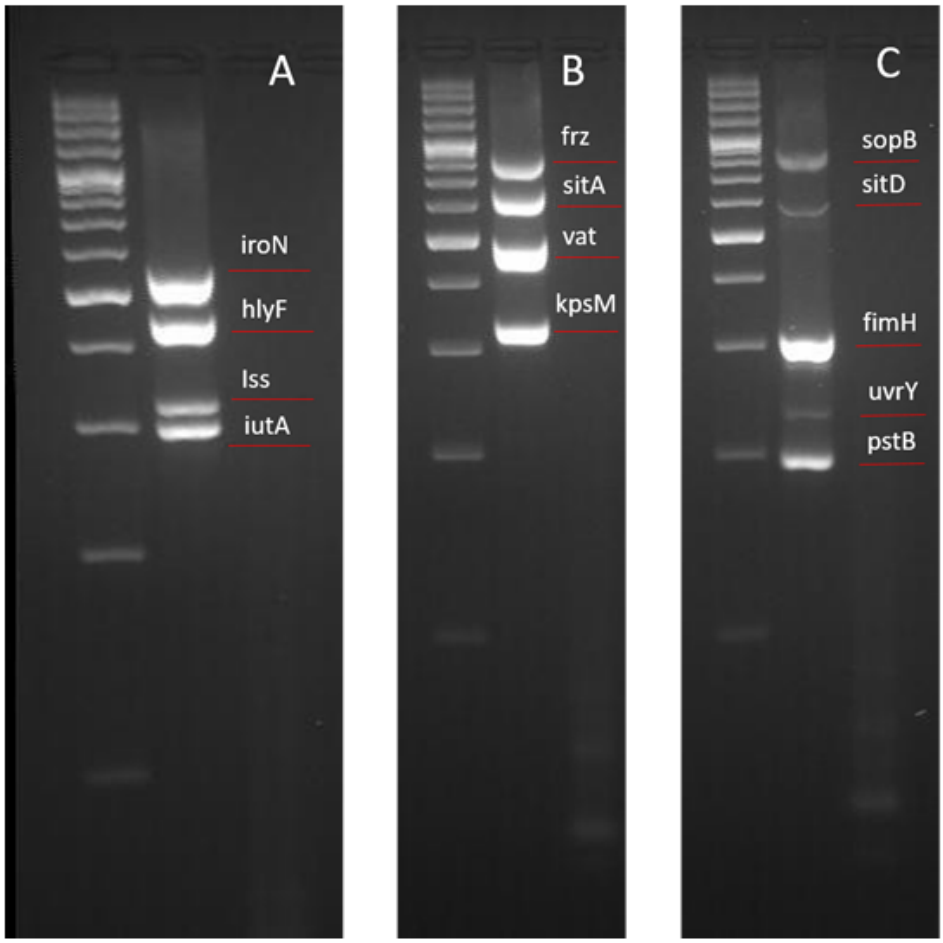

Figure 1

Multiplex PCR. Amplification bands for each of the multiplex PCR, (A) Multiplex 1, *iroN* 553 bp, *hlyF* 450 bp, *Iss* 332 bp and *iutA* 302 bp. (B) Multiplex 2, *frz* 843 bp, *sitA* 661 bp, *vat* 498 bp and *KpsM* 335 bp. (C) Multiplex 3, *sop*B 797 bp, *sitD* 571 bp, *fimH* 331 bp, *uvrY* 286 bp and *pstB* 198 bp. The gels were prepared at 4% agarose and a 100 bp weight marker was used.

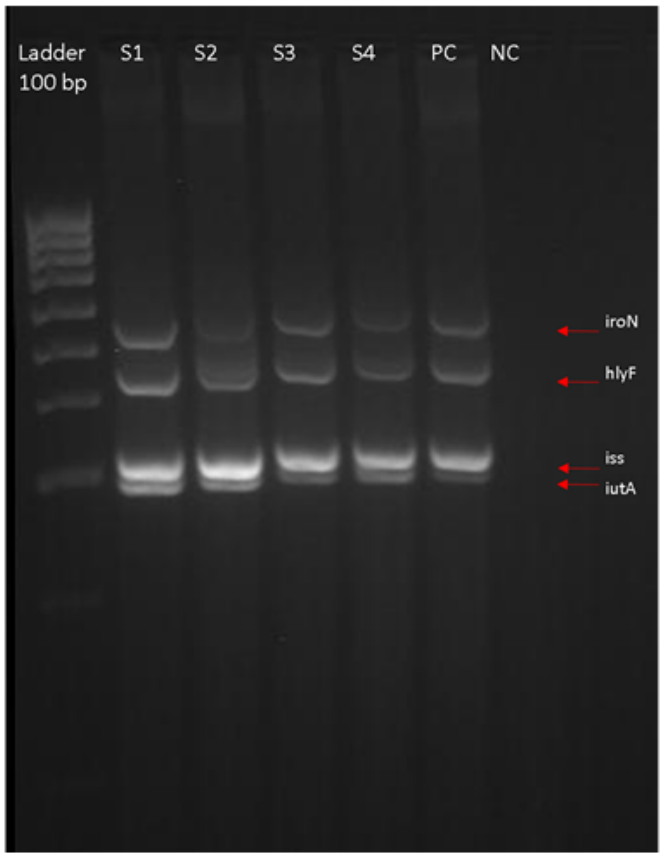

Figure 2

Multiplex PCR 1. The gel image shows one of the runs of multiplex 1, for 4 samples of isolates. From left to right: 100 bp weight marker, samples coded from S1 to S4, positive control (PC) and negative control (NC). The red arrows indicate the corresponding position to the band of the amplicons of each of the genes (*iroN*, *hlyF*, *Iss* and *iutA*). The gel was prepared at 4% agarose.

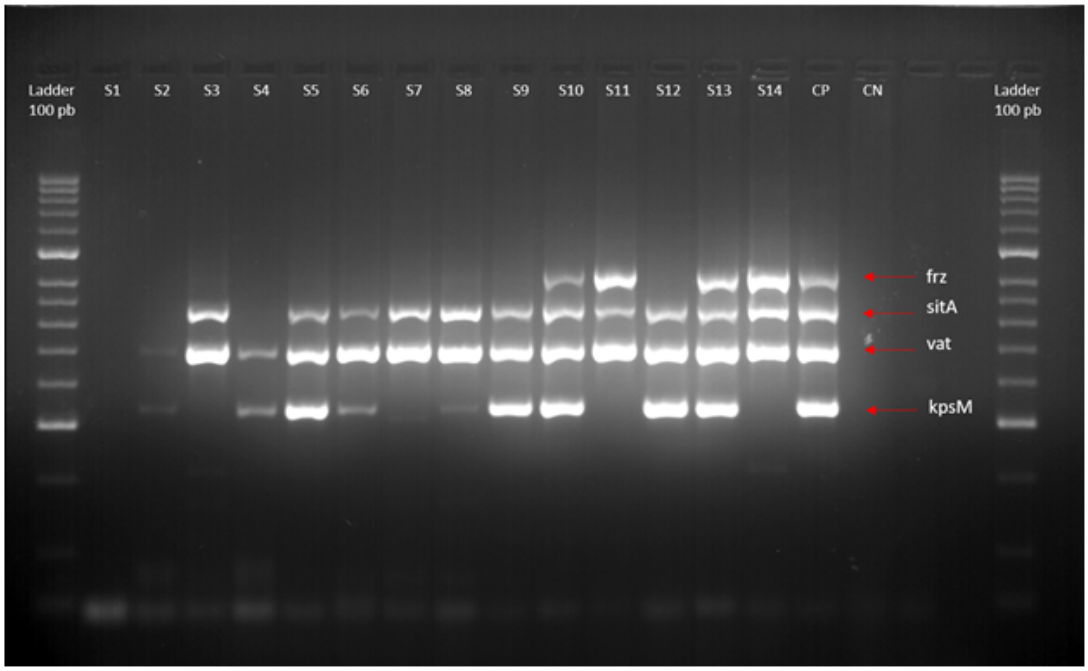

Figure 3

Multiplex PCR 2. The gel image shows one of the runs of multiplex 2, for 14 samples of isolates. From left to right: 100 bp weight marker, samples coded from S1 to S14, positive control (CP), negative control (CN) and 100 bp weight marker. The red arrows indicate the corresponding position to the band of the amplicons of each of the genes (*frz*, *sitA*, *vat* and *KpsM*). The gel was prepared at 4% agarose.

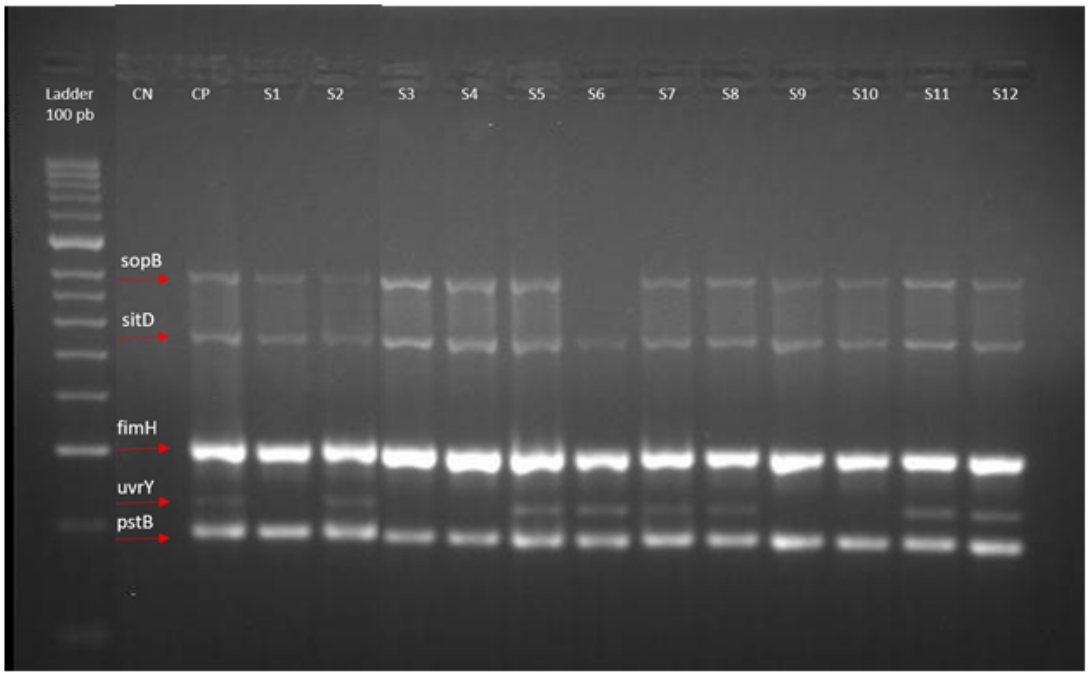

Figure 4

Multiplex PCR 3. The gel image shows one of the runs of multiplex 3, for 12 samples of isolates. From left to right: 100 bp weight marker, negative control (CN), positive control (CP) and samples coded from S1 to S12. The red arrows indicate the corresponding position to the band of the amplicons of each of the genes (*sop*B, *sitD*, *fimH*, *uvrY* and *pstB*). The gel was prepared at 4% agarose.