RESEARCH REPORT
SWINE HEALTH

Title: Development and validation of molecular-based tools to differentiate attenuated *Salmonella choleraesuis* vaccine strains from field isolates - **NPB #: 09-094**

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Industry Summary:

The objective of this research was to develop molecular diagnostic methods for differentiating live, attenuated *Salmonella* Choleraesuis vaccines from virulent *Salmonella*-like bacteria.Samples submitted to the Minnesota Veterinary Diagnostic Lab (MVDL) as part of routine diagnostic workup were used for test development. 115 suspected vaccine isolates and 101 known *Salmonella* Choleraesuis isolates were tested using the newly developed test. 22 additional *Salmonella* serotypes and 14 unrelated pathogens were also tested to demonstrate the specificity. The multiplex test for the Argus live vaccine strain of *Salmonella* Choleraesuis was successful in quickly and specifically identifying the Argus vaccine and in differentiating it from virulent strains of *Salmonella* Choleraesuis. This test is currently being offered for routine diagnostics at the Minnesota Veterinary Diagnostic Laboratory.

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Scientific Abstract:

The Salmonella enterica serovar Choleraesuis attenuated Argus vaccine strain is currently used in the United States as an aid to prevent and control infection by this pathogen in swine herds. Similarly to virulent Salmonella Choleraesuis field isolates, the attenuated Argus vaccine strain can be isolated from systemic sites in vaccinated pigs. The objective of this study was to develop a molecular based diagnostic tool for the rapid and accurate identification of the S. Choleraesuis Argus vaccine strain isolated from systemic sites of vaccinated pigs. Primers targeting the *crp* gene of S. Choleraesuis were designed and combined with previously published

primers targeting the *fli*C gene in a multiplex PCR reaction. The multiplex PCR was validated using DNA extracted from isolates representing 19 *Salmonella* serotypes, 14 unrelated swine bacterial pathogens commonly isolated from swine tissues, 115 suspect Argus vaccine isolates and 101 field isolates recovered from clinical cases identified as S. Choleraesuis. The absence of a *crp* band and the presence of a *fli*C band (- crp/ + *fli*C) were uniquely identified in the Argus vaccine strain. *Salmonella* Choleraesuis isolates were identified based on the presence of both *crp* and *fli*C bands (+ crp/ + *fli*C), whereas additional *Salmonella* serovars were characterized by the presence of a *crp* band and absence of the *fli*C band (+ crp/ - fliC). None of these profiles were observed in non-*Salmonella* swine bacterial pathogens. The Argus vaccine profile (+fliC/ - crp) was not detected in 101 field S. Choleraesuis isolates. This profile was identified in 113/115 6,7:non-motile isolates recovered from herds using this vaccine.

Introduction:

Salmonella enterica serovar Choleraesuis is an important cause of systemic disease and mortality in swine. This serovar has also been linked to human infections resulting in septicemia and occasional death (Jean et al, 2006). Isolates recovered from swine and human cases tend to be highly resistant to commonly used antibiotics (Chiu et al, 2004; Hsu, 2006). In light of the increasing antibiotic resistance, vaccination has become an important alternative for disease control.

Live attenuated S. Choleraesuis vaccines are reportedly effective in reducing prevalence of infection and mortality associated with systemic infection (Curtis et al, 2003). The Argus S. Choleraesuis vaccine, for example, reportedly reduces the prevalence of infection in endemically infected populations (Maes et al, 2001). Similarly to virulent S. Choleraesuis field isolates, the Argus vaccine strain can invade extra-intestinal tissues and be isolated from systemic sites (Loynachan et al, 2004). One of the main limitations of such vaccines, however, is the lack of diagnostic tools available to differentiate vaccine from virulent field isolates.

The S. Choleraesuis Argus vaccine is attenuated as a result of deletions in the adenylate cyclase (*cyaA*) and cAMP receptor protein (*crp*) genes. These mutations affect the transport and catabolism of carbohydrate, amino acid and peptides, in addition to glycogen synthesis. As a result, the Argus S. Choleraesuis strain does not produce H_2S and has a sugar fermentation profile considerably different from other S. Choleraesuis. Due to these modifications, this vaccine strain is often misidentified as *Hafnia alvei* in clinical diagnostic laboratories (Curtis et al, 1993).

The objective of this study was to developed and validate a rapid and accurate multiplex PCR test to identify the Argus vaccine strain and differentiate it from virulent S. Choleraesuis field isolates.

Objectives:

Salmonella choleraesuis live vaccines are widely used by the swine industry for disease prevention and control. Although isolates in these vaccines are attenuated, they may still invade the host and be isolated from systemic tissues. Herds utilizing live *S. choleraesuis* vaccines often request that diagnosticians identify if the *S. choleraesuis* isolates being recovered from systemic tissues are field or vaccine strains. The tools needed for this differentiation are currently unavailable.

In this study, we will develop and validate molecular-based diagnostic tools to differentiate between vaccine and field strains of *S. choleraesuis*. During the last year we have gathered relevant diagnostic information demonstrating that one of the most used attenuated *S. choleraesuis* vaccines available in the market cannot be differentiated from field isolates circulating among U.S. swine herds based on phenotype, biochemical testing, genomic fingerprint, and antibiotic susceptibility.

There is a critical need to develop and validate the necessary diagnostic tools to differentiate between field and vaccine *S. choleraesuis* strains. This knowledge is important for swine veterinarians and producers, as it will

define if isolation of *S. choleraesuis* from systemic tissues is due to actual clinical disease or the result of vaccination.

Materials and Methods:

Isolates. The S. Choleraesuis vaccine strain was cultured directly from the commercial product (Argus® SC/ST Schering-Plough, USA). Field isolates used in this study were obtained from routine tissue submissions at the University of Minnesota Veterinary Diagnostic Laboratory (MVDL). Group C1 *Salmonella* Choleraesuis and 6,7:non-motile Salmonella-like (*Hafnia alvei*) isolates were identified based on biochemical profiles and serotyping at the National Veterinary Services Laboratories (NVSL). A total of 101 S. Choleraesuis and 113 Salmonella-like field isolates were used to validate the multiplex assay.

DNA extraction. *Salmonella* Choleraesuis and 6,7:non-motile Salmonella-like isolates (suspect Argus vaccine isolates) were cultured on 5% sheep blood agar (SBA) and incubated aerobically at 37°C during 24 hours prior to DNA extraction. *Salmonella* colonies were harvested from the SBA plates, suspended in pH 7.2 phosphate buffered saline solution and centrifuged at 28,350 x g during 3 min to form a pellet. The supernatant was discarded and the pellet was suspended in 200µl PrepMan Ultra Sample Preparation Medium (Applied Biosystems). The suspension was boiled for 20 minutes and was centrifuged at 28,350 x g during 3 min. The supernatant containing extracted DNA was transferred to a 0.6 ml tube and was diluted 1:1 in RNase/DNase-free water. Extracted genomic DNA was stored at -20° C until used.

***cya*A and *crp* amplification and sequencing.** The literature reports that the Argus vaccine contains deletions in the *cya*A and *crp* genes(Kelly et al, 1992); however, the characteristics of these deletions are not fully disclosed. In order to better characterize signature mutations in the Argus vaccine strain, universal primers were designed to amplify and sequence the *cya*A and the *crp* genes in *Salmonella* sp. The following primers were used: *cya*AF: 5'-GCCATAAATCAACTGCGT GT - 3', *cya*AR: 5'-TCAGGAGAAATACTGCTGCAA-3', *crp*F: 5'-GCAAACAGACCCGACTCT TG-3', *crp*R: 5'-GGTGCCGTAGACGACGAT-3'. PCR reactions to amplify each target gene contained 12 µl QIAgen HotStarTaq MasterMix, 10.4 µl QIAgen RNase-free H2O, 0.8 µl each of 10 µM forward and reverse primers, and 1 µl extracted DNA (approximately 100 ng). Optimal annealing temperatures were determined for each primer set by running an annealing gradient experiment. The final PCR cycling conditions were as follows: initial denaturation at 95° for C 15 min followed by 30 cycles of denaturation at 94° C for 30 sec, annealing at 60° C for 1 min, extension at 72° C for 3 min, final extension at 72° C for 10 min, and a final hold of 4° C. PCR products were analyzed for presence of amplicons with expected sizes (*cya*A 2508 bp and *crp* 610 bp) by gel electrophoresis on a 1% agarose gel containing 0.5µg/ml ethidium bromide and visualized under UV light. Once an amplicon was detected, the remaining PCR product was purified using the QIAgen QIAquick PCR Purification kit following the manufacturer's protocol and sequenced at the University of Minnesota Biomedical Genomics Center. Sequencing reactions consisted of 9 µl RNase-free water, 2ul of a 10 µM forward or reverse primer, and 60 ng of purified DNA. Obtained sequences were identified by performing a BLAST search at the GenBank (Altschul et al, 1990). Sequences were also aligned and analyzed using MEGA 4.1 software (Tamura et al, 2007).

Multiplex PCR. Based on results obtained following detection and sequencing of signature mutations in the Argus vaccine strain, the *crp* gene was selected to be included in the multiplex PCR. *Salmonela* Choleraesuis species-specific primers targeting the *fli*C gene were included to add specificity to the multiplex test. (Figure 1). The following primers were used in the multiplex reaction: crpF and crpR (designed in this study), *flin*C-F: 5'-AAG GAA AAG ATC ATG GCA CAA-3', *flin*C-R: 5'-GAA CCC ACC ATC AAT AAC TTT G-3' (previously published, (Chiu et al. 2005). PCR reactions consisted of 2 ul of the extracted DNA added to 48 ul of PCR mix containing 25 µl 2x QIAgen Multiplex MasterMix, 5 µl 10X primer mastermix containing 10 µM

of each primer (*crp*F, *crp*R, *flin*C-F, *flin*C-R), and 18 µl RNase-free water. The cycling conditions were as follows: 15 min activation step of 95° C, 30 cycles of denaturation at 94° C for 30 sec, annealing at 60° C for 90 sec, extension at 72° C for 90 sec, a final extension step of 72° for 10 min, followed a hold at 4°C. PCR products were analyzed on 1% agarose gel containing 0.5 µg/ml ethidium, and visualized under UV light. Expected sized for *crp* and *fli*C amplicons were 610 bp and 963 bp, respectively.

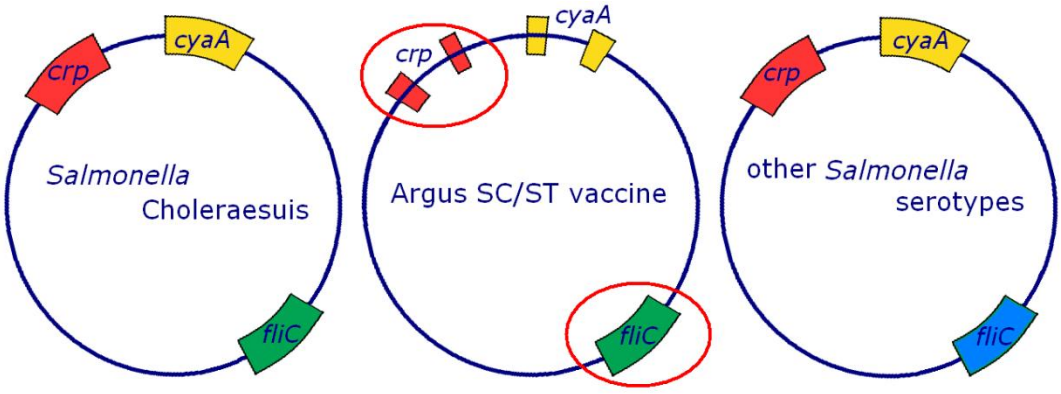

Figure 1. Selected genetic differences between *Salmonella* Choleraesuis (A), Argus SC/ST vaccine (B), and other *Salmonella* serotypes (C) used for multiplex test development. The unique mutation in the Argus vaccine strain *crp* gene and the differences in *fli*C gene among *Salmonella* serovars were used to develop the multiplex PCR test.

Results:

Detection and sequencing of *cyaA* and *crp* genes. Both crp and cyaA primers developed in this study successfully amplified the target genes in S. cholerasuis with the expected amplicon sizes (610 and 2508 bp, respectively). Amplification of both genes in S. sholeraesuis was confirmed by sequencing. These primers failed to amplify the *crp* and *cya*A genes in the Argus vaccine strain, and no sequence information on potential signature mutations were obtained for this strain.

Multiplex PCR. Primers targeting the *crp* gene of S. Choleraesuis was selected to be included in the multiplex PCR in addition to previously published S. Choleraesuis-specific primers for detection of the flagellar antigen *fli*C gene (Chiu et al. 2005). The multiplex test was validated using 101 *S.* Choleraesuis isolates, 113 *Salmonella*-like 6,7:nonmotile field isolates, 22 serotypes of *Salmonella enteric* other than Choleraesuis (Table 1) and 14 non-Salmonella bacterial species commonly isolated from swine (Table 2). All Salmonella Choleraesuis isolates (n=101) produced the expected double band profile (+ crp/ + fliC), whereas 113 of 115 Salmonella-like 6,7:non-motile isolates generated the expected profile for the Argus vaccine strain (- crp/ + fliC) (Figure 2). Two Salmonella-like 6,7:nonmotile isolates produced atypical results compared with the majority of the isolates tested, producing + crp/ - fliC and + crp/ + fliC profiles respectively. *Salmonella* serovars Choleraesuis (n=22) were characterize by the presence of a *crp* band and absence of the *fli*C band (+ crp/ - fliC). None of these profiles were observed in non-*Salmonella* swine bacterial species (n=14). Results are summarized in Table 3.

Confirmation of vaccination status: After establishing the validity of this test for the differentiation of *Salmonella*-like 6,7: non-motile isolates from *Salmonella* Choleraesuis, the herd vaccination status was verified for all positive results and isolates typed as 6,7: non-motile, to confirm that this multiplex PCR was detecting the Argus vaccine strain rather that other atypical *Salmonellae*. Vaccination status was available for 43 isolates. 41 of the 43 6,7: nonmotile isolates were confirmed to have used the Argus vaccine at the time that cases were submitted to the MVDL. Of these, all tested positive with this multiplex PCR. One of the remaining isolates

originated from a herd which did not use the Argus vaccine at the time of submission to the MVDL, while the other came from a herd vaccinating with the Argus vaccine.

Table 1. Specificity of Argus multiplex test (flinC and crp primers) on different salmonella serotypes

***Salmonella* Serotype**	**Serogroup**	**Amplicons**	**PCR Result**
Choleraesuis	C1	610bp/963bp	-
Argus	C1	963bp	+
Krefeld	E	610bp	-
Worthington	G	610bp	-
Braenderup	B 4,5	610bp	-
Brandenburg	B 4,5	610bp	-
St. Paul	B 4,5	610bp	-
Dublin	D1	610bp	-
Enteritidis	D1	610bp	-
Derby	B 4,5	610bp	-
Typhimurium (copenhagen)	B 4,5	610bp	-
Agona	B 4,5	610bp	-
Newport	C2	610bp	-
Mbandaka	C1	610bp	-
Montevideo	C1	610bp	-
Tennessee	C1	610bp	-
Hartford	C1	610bp	-
Thompson	C1	610bp	-
Bareilly	C1	610bp	-
Infantis	C1	610bp	-
Ohio	C1	610bp	-

Table 2. Specificity of Argus multiplex test (flinC and crp primers) on unrelated pathogens

Species	**Amplicons**	**PCR Result**
Haemophilus parasuis	No Amplification	-
Actinobacillus porcinus	No Amplification	-
Actinobacillus suis	No Amplification	-
Bordetella bronchiseptica	No Amplification	-
Streptococcus suis	No Amplification	-
Actinobacillus minor	No Amplification	-
Actinobacillus indolicus	No Amplification	-
Pasteurella multocida	No Amplification	-
Actinobacillus pleuropneumoniae	No Amplification	-
Brachyspira pilosicoli	No Amplification	-
Escherichia coli	-	-
Leptospira hardjo	No Amplification	-
Clostridium perfringens	No Amplification	-
Mycoplasma hyorhinus	No Amplification	-
Mycoplasma hyopneumoniae	No Amplification	-

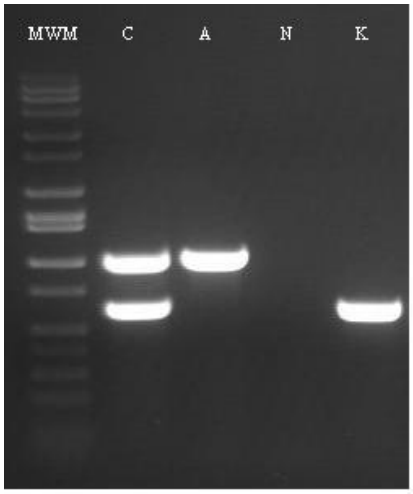

Figure 2. Multiplex PCR results. MWM=molecular weight marker, C=*Salmonella* Choleraesuis, A= Argus vaccine, N=negative control, K*salmonella* Krefeld

Table 3. Summary of multiplex PCR results

Multiplex PCR profile	S. Choleraesuis	6,7:non-motile	Total
Argus Vaccine (+ *fli*C/ - *crp*)	0	113	113
Salmonella Choleraesuis (+ *fli*C/ + *crp*)	101	1*	102
Other Salmonella Serotypes (- *fliC/* + *crp)*	0	1*	1
Total	101	115	216

Discussion:

Because live attenuated *Salmonella* vaccines can be isolated from systemic tissues, these vaccine strains must be distinguished from their parent strains and other atypical *Salmonella* strains, as correct diagnosis is important for herd vaccination monitoring and epidemiological studies. While the attenuating mutations in the Argus vaccine cause a change in phenotype, proper identification requires two steps, traditional bacteriology and serotyping, which can be very time consuming and expensive. The multiplex test that we have developed can produce an accurate identification in several hours and provides a more specific identification.

The validation of this test demonstrated the improved accuracy of this test. 3 *Salmonella*-like isolates initially serotyped as Choleraesuis, 1 isolate initially serotyped as ohio, and 2 initially serotyped as Bareilly tested positive by our multiplex PCR test, but after they were resubmitted for serotyping, they were confirmed as 6,7: non-motile, the serotype of the Argus vaccine. Additionally, one *Salmonella sp.* isolate initially serotyped as 6,7: nonmotile produced a negative PCR result and was confirmed to be Choleraesuis after resubmitting for

serotyping. And one isolate typed as Choleraesuis produced a single crp band which should represent something other than Choleraesuis, and this was found to be serotype Thompson after repeating serotyping.

Additionally, two *Salmonella sp.* serotype 6,7: nonmotile isolates produced PCR results which conflicted with the serotyping results which were not resolved by repeating serotyping, and provide examples of the advantage of using molecular markers rather than serotyping. In the first case, the isolate was serotyped as 6,7: nonmotile, but the PCR test produced a single 610 bp band indicating that is was *Salmonella sp.* other than *S.* Choleraesuis. It is hypothesized that this isolate represents a wild strain of *Salmonella* serotype 6, 7: nonmotile. Wild strains of this serotoype have been isolated from swine lymph nodes and have been shown to experimentally infect swine (11). This is consistent with our finding that the isolate originted from a pig in a herd not using the Argus vaccine, and it is unlikely that the vaccine would have spread from another herd. Additionaly, the isolate was typed as *Salmonella sp.* rather than *Salmonella*-like by traditional bacteriological identification.

In the second case, the isolate was serotyped as 6, 7: nonmotile, but the PCR test produced a double band indicating that it was *Salmonella* Choleraesuis. It is believed that this isolate is in fact a misidentified *S.* Choleraesuis isolate. This isolate produced the flinC amplicon in our test, which was previously demonstrated to be specific to serotypes Choleraesuis and Paratyphi C.Chadfield et al (2002) reported a number of *Salmonella* isolates (serotypes Tennessee, Infantis, and Livingstone) which were misidentified as serotype 6,7: nonmotile due to suppressed motility and were correctly identified only after more in-depth motility assays (2). . *S.* Choleraesuis differs from serotype 6, 7: nonmotile in its expression of flagella (6) so it is likely that a failure to detect the flagella could lead to misidentification in our case as well.

The *crp* gene should make a very good, stable molecular signature for this test. The Argus vaccine was attenuated by creating a presumably large deletion mutation to the *crp* gene, which normally plays an important role in a wide range of cellular functions, so it is unlikely that this mutation would occur naturally, especially in light of the fact that mutations to the *crp* gene have detrimental effects on the organism, including metabolism and motility (7). The chances of picking up other atypical *Salmonellae* based on this marker are unlikely.

Despite the strengths of this molecular marker, using the absence of the *crp* gene alone in a singleplex PCR could potentially produce misleading results, because unrelated swine pathogens would also lack this *crp* amplicon. The flinC primers add another level of specificity. This *S.* Choleraesuis-specific primer set was designed by Chiu et al (2005) (3), and targets a hypervariable region of the flagellar protein antigen in *S.* Choleraesuis. Because the Argus vaccine was derived from *S.* Choleraesuis by a targeted mutagenesis, it was hypothesized that the region of the *fliC* gene targeted by the flinC primer, which is specific for *S.* Choleraesuis, would remain unchanged in the Argus vaccine strain. This was confirmed, as this fragment was present in 100% of *S.* Choleraesuis and 100% of Argus vaccine isolates during validation of the multiplex test, but absent in all serotypes and non-salmonella isolates tested, and by those cited in the publication. Combined with the crp primer set, this multiplex test is specific for identifying the Argus vaccine.

High specificity and reliability of this test were proven by validation, and the use of a gel-based PCR protocol is fast, and cost-effective. This test will provide a valuable diagnostic tool for veterinarians to identify the Argus vaccine, helping to monitor salmonella control through monitoring of vaccination, and helping to identify and treat atypical salmonella cases.

Further experiments could be conducted to determine the sensitivity of this test and see if it can accurately identify the Argus vaccine from tissue or stool samples, which could potentially expedite the process.

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