4 Results

4.1 Establishment of a chicken infection model

4.1.1 Clinical findings

The first step in establishing a Signature-tagged mutagenesis (STM) approach is the establishment of an appropriate infection model, which is able to give a high enough discriminative read out between attenuated and non-attenuated mutants. To estimate the range of challenge doses, published data on APEC infections via air-sac inoculation were taken into consideration. Inoculated chickens received doses ranging from 10^5 to 10^9. Those animals receiving between 10^8 and 10^9 CFU bacteria were depressed and reluctant to move, simultaneously demonstrated head shaking and a rough feathering appearance as early as 6 h post inoculation. At 12 h post inoculation, these chickens manifested mild respiratory sounds. All chickens from the group receiving 10^9 CFU were dead or moribund at 48 h post inoculation, whereas only half of chickens from 10^8 CFU group were dead. Between strains IMT5155 Nal^R and its wild type strain IMT5155, no differences of virulence were observed. In contrast, none of the chickens receiving lower doses died. Control chickens remained clinically normal at all times.

4.1.2 Pathological findings

Six hours after inoculation, mild to moderate air sac lesions were observed in chickens from the groups with the dose of 10^8 bacteria. At 12 hours postinoculation, moderate to severe bilateral airsacculitis, affecting the caudal thoracic and adjacent air sacs, were present in all chickens infected with the dose of 10^8 CFU bacteria. Blood appeared to coagulate easily in these birds. Twenty-four hours after inoculation, severe bilateral airsaculitis, moderate pericarditis, marked pulmonary congestion and severe hypertrophy and congestion of the spleen, with multiple petechial hemorrhages presented in muscles, subcutaneous tissues, visceral fat tissues and serous surfaces, were observed in these chickens (Fig. 5).

Fig. 5a

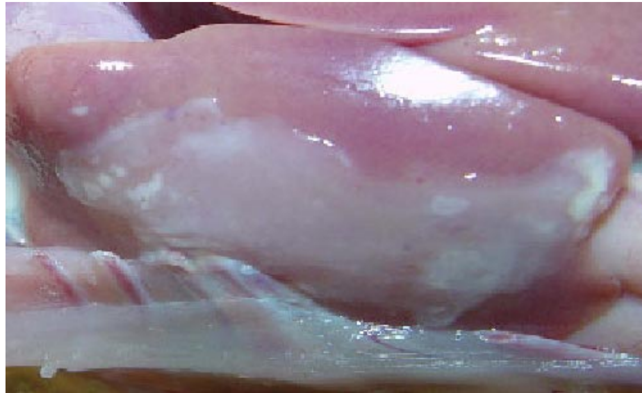


Fig. 5b

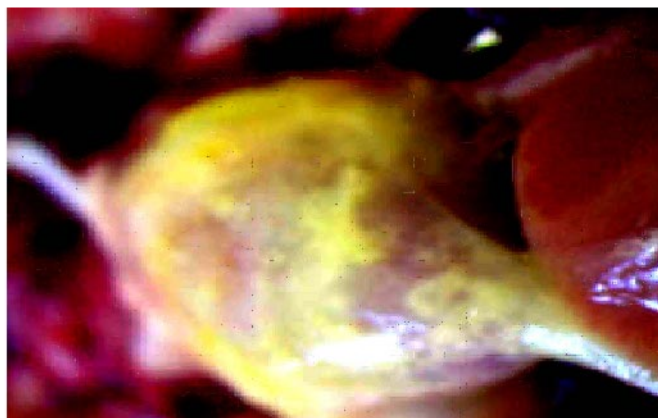


Figure 5: Patho-macroscopical findings in 5-week-old chickens intratracheally inoculated with APEC IMT5155 Nal^R. Reproduction of perihepatitis **(a)**, and pericarditis **(b)**

At 48 h post inoculation, lesions were similar but more marked, with the added presence of mild to moderate perihepatitis covered by a thick yellow fibrous membrane. No difference was found between chicken groups infected with *E. coli* IMT5155 Nal^R and its wild type

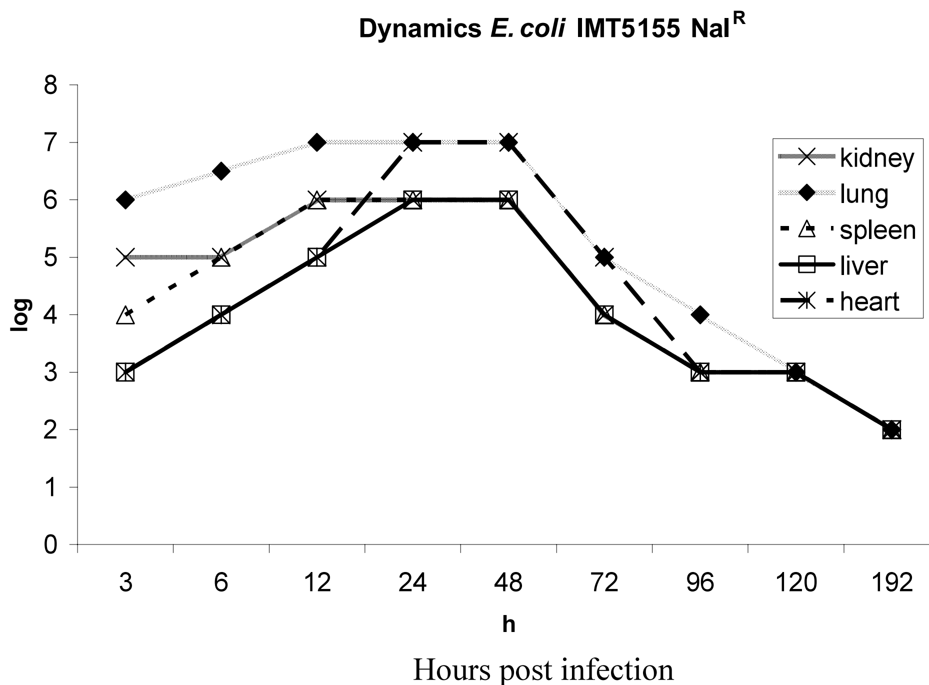
IMT5155. No lesions were observed in chickens from groups infected with *E. coli* IMT5104.

Due to the findings that APEC disease could be reproduced with these initial experiments, a dose of 10^8 CFU was chosen for further experiments.

4.1.3 Systemic spread of infection

Another crucial step in the establishment of an in vivo STM approach is knowledge about the dynamics of the bacterial load in defined organs. To study the dynamics of IMT5155 Nal^R in vivo, 60 five-week-old chickens were infected with a dose of 10^8 CFU bacteria and killed at different intervals.

Bacterial load (log CFU)



Hours post infection

Figure 6: Quantitative bacterial counts in lung, heart, liver, spleen and kidney at different times after inoculation of 60 chickens with APEC strain IMT5155 Nal^R. The CFUs are the average of results gained with six chickens.

Bacteria appeared rapidly in lung, liver, heart, spleen and kidney. A great increase in the bacterial counts was observed from 12 to 48 h, with counts of individual birds being as high as 7 to 8 log CFU/g (Fig. 6). The percentage of chickens with colonized lung, heart, liver, spleen and kidney was about 85%. After 48 h, the counts in the internal organs dropped dramatically, although still as much as 2 log CFU/g IMT5155Nal^{R} could be detected from lung and other internal organs.

4.2 Establishment of a STM-mutant bank

The method of signature-tagged transposon mutagenesis was developed by Hensel et al. (1995). The tag consists of a short DNA sequence that is composed of two arms and one 40 bp central region. The central region is unique for each tag while the arms which flank the central region are identical and are used for amplification of the central region by PCR. When the tagged transposons are used to mutagenize an organism, each individual mutant can in theory be distinguished from any other mutant based on the different tags carried by transposons that inserted in one site of its genome.
The STM strategy applied in this study was modified in order to use a non radioactive detection method, namely a DNA-DNA dot blot instead of the radioactive colony blot. In this improved version of STM, a series of tagged transposons are selected before mutagenesis of the respective pathogen, based on efficient tag amplification and labelling and lack of cross-hybridization to other tags. These selected transposons are then used separately to generate a large number of bacterial mutants that are arrayed based on the tags they carry.

4.2.1 Prescreening of 96 transposons with unique tags

pUT-mini-Tn5*km2* containing signature tags were transformed into *E. coli* CC118 λpir by electroporation, in which 1 µg of plasmid DNA gave approximately 7×10^8 transformants. 96 of these transformants were then picked up to form a new pool, and plasmid dot blots hybridization was performed with their corresponding digoxigenin-dUTP-labeled probe. The results showed that approximately one-fourth of plasmids could give strong signal when hybridization with their cognate labelled tags, one-eighth of the plasmids gave absolutely no signal, and five-eighth of them only yielded a very weak signal (Fig. 7a). Therefore, a total of 384 transformants were grouped in four sets of 96 each. These four pools were screened by plasmid dot blot hybridization with their corresponding digoxigenin-dUTP-labelled probes. Plasmids which gave strong signals after hybridization were picked up to form a new pool containing 96 transformants (Fig. 7b).

Fig. 7a

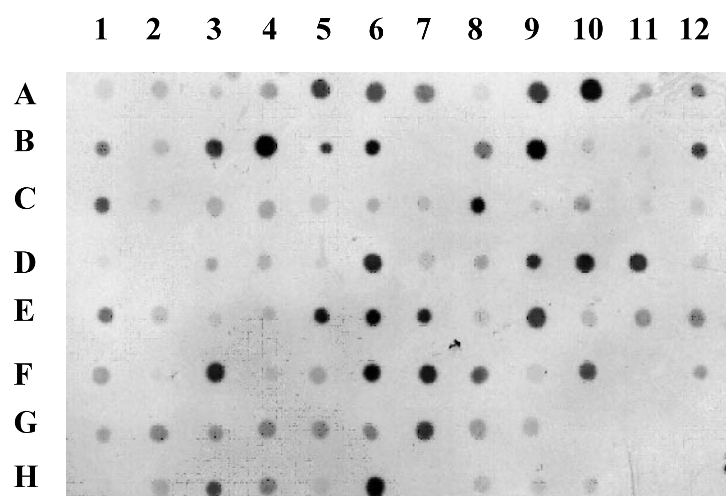


Fig.7b

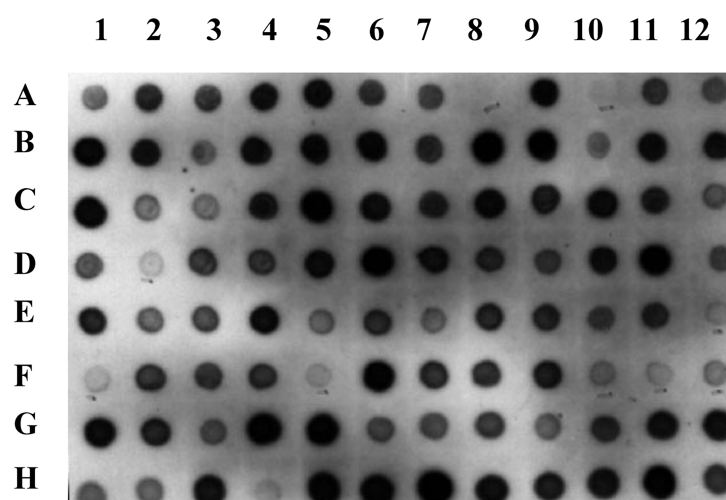


Figure 7: Dot blot hybridization for prescreening of 96 signature tags. **a)** approximately one-fourth of the tags gave strong signal when hybridized with their corresponding digoxigenin-dUTP-labeled probes. **b)** 96 tags selected from 384 transformants gave strong signal when hybridized with their corresponding digoxigenin-dUTP-labeled probes.

4.2.2 Improvements for non-radioactive use

In our initial hybridization experiments, Digoxigenin-labelled probes were used in dot blots. The target DNA consisted of 100 ng of each preselected tagged pUTmini-Tn5*km2*.

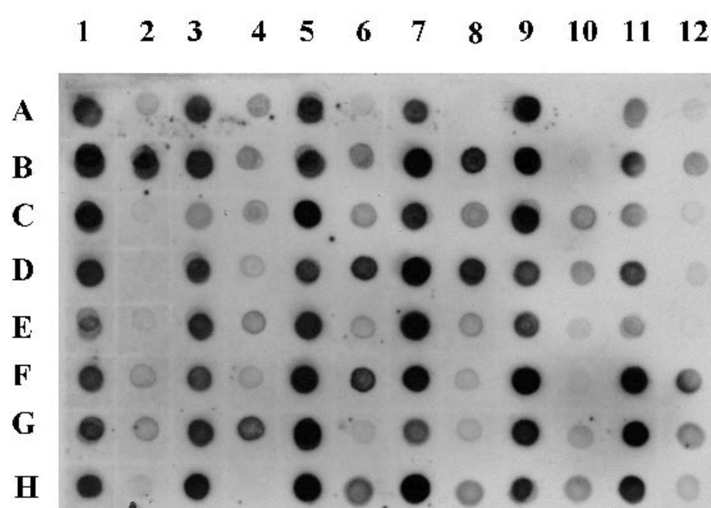
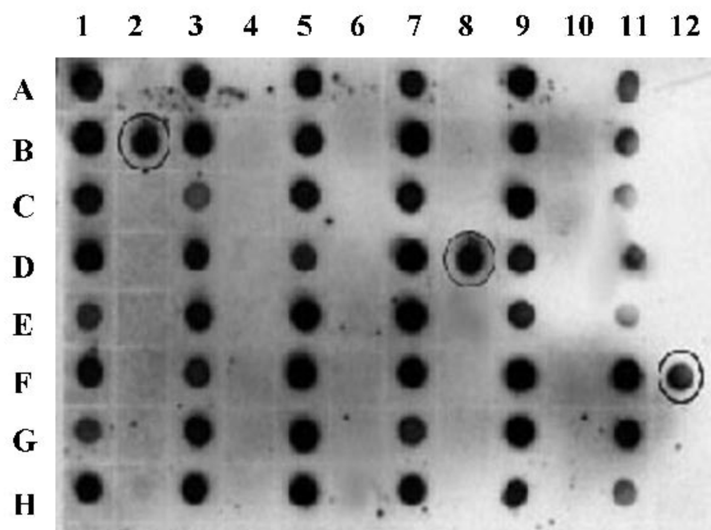


Figure 8: Probes for each dot blot were generated by combining the $[NK]_{20}$ tagged plasmids from each of the corresponding target DNAs in line 1, 3, 5, 7, 9 and 11. Blotting procedures differed from Fig. 9 with respect to the type and quantity of target DNA applied to the membrane, amount of dig-dUTP used for amplification and final purification of the probe: target DNA was 30 ng of each preselected $[NK]_{20}$ plasmid. Probes were labelled with DIG-dUTP and digested with *Hind*III but not purified from the constant primer arms.

Probes were labelled with DIG-dUTP using the Roche Labeling and Detection Kit (Roche, Mannheim, Germany) as recommended by the vendor, utilizing primers P2 and P4. Subsequently probes were digested with *Hind*III but were not purified from the constant primer arms. This strategy gave unacceptable background levels of hybridization in negative controls (Fig. 8). In consideration of background caused mainly by constant primer arms, background was reduced by increasing the digestion level of *Hind*III.

a)



b)

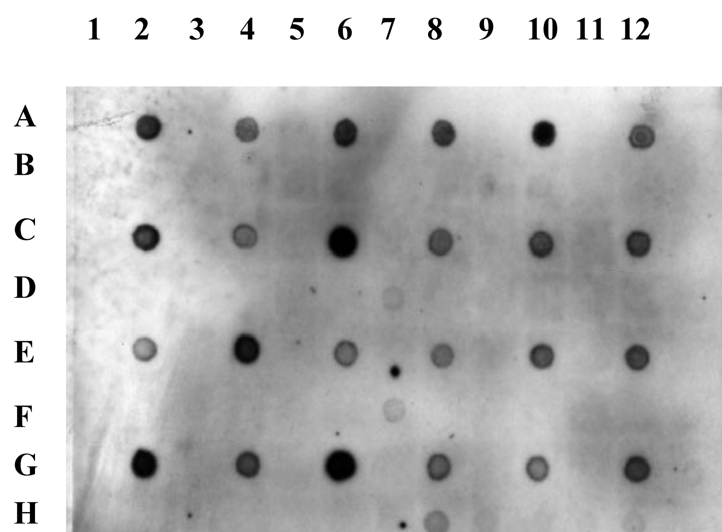


Figure 9: **a)** 30 ng of each PCR-amplified $[NK]_{20}$ tag from the preselected plasmids served as target DNA. Probes were prepared with half of the standard concentration of DIG-dUTP as mentioned in (Fig. 8), digested with *Hind*III and were gel purified from the constant primer arms before use. Cross-hybridization with other tags was detected in B2, D8 and F12. Thus, these tags were excluded from the selected plasmids. **b)** The same was done with tags D7, F7 and H8.

A lower concentration of DIG-dUTP was used in the PCR reaction for probe labelling to ensure more complete digestion by *Hind*III. This strategy indeed reduced the background. On the other hand, lower levels of dig-dUTP labelling also led to weaker signals when visualized in a certain exposure time. In order to obtain clear signals, a 1:1 mix of DIG Probe Synthesis Mix with 2.5 mM dNTP stock solution was used in the labelling PCR reaction. In addition, the conserved arm sequences of tags were separated by gel electrophoresis. Briefly, the probes were labelled by half of concentration of DIG-dUTP, then digested by *Hind*III in a volume of 400 µl, subsequently purified by running a 4 % 3:1 NuSieve GTG : agarose gel electrophoresis. Only the band of 40 bp DNA was excised from the agarose gel and directly used as probe in hybridization experiments. The most significant reduction in background of hybridization was achieved by generating tag sequences with PCR as the target DNA for dot blots rather than using the entire plasmid containing the tagged transposon. As shown in Fig. 9, these protocol modifications were able to eliminate a significant amount of background making chemiluminescent/nonradioactive screening more effective.

4.2.3 Cross-hybridization among selected tags

The sequences of signature tags were designed to ensure that the same sequence should only occur once in a total of 2×10^{17} molecules. Although the likelihood of duplicated tags is extremely low, any possible cross-reaction among these selected 96 tags were explored. Half or one–fourth of the plate of master tags were collected and labelled by the improved method, then probed against the whole set of tags to further demonstrate if tags were duplicated. The results are shown in Fig 9ab. A total of six tags showed strong cross-hybridization with other tags. Thus, these six tags were excluded from selected tags. Therefore, 90 tags which were amplified and labelled easily and had not cross-hybridization were retained for further research.

4.2.4 Construction of the mutant library

A library of 1.827 signature-tagged mini-Tn5*km2* mutants of strain IMT5155 was constructed according to the methods of Hensel *et al.*, (1995) using 90 prescreened non-cross-reactive tags that could be amplified and labelled efficiently as described in experimental procedures. Nineteen of the mutants (about 1 %) were ampicillin resistant, indicating the presence of the suicide plasmid containing the transposon within the cytoplasm or integrated into the chromosome. Besides, eight mutants lost the kanamycin resistance when they were recovered from - 70ºC, indicating that they had lost the transposon spontaneously. These mutants were eliminated from the library. In order to detect if the transposon had any hot spot for insertion, southern blotting analysis were performed on eighteen randomly chosen mutants. Blots were prepared containing genomic DNA from these 18 mutants digested with *Pst*I, and probed with a fragment of the kanamycin resistance gene amplified from the pUT-mini-Tn5*km2* by primer pair P19 and P20. The hybridization pattern from each *Pst*I-restricted mutant differed from one another and each mutant showed only one positive band (data not shown). Overall, these tests indicated a broad distribution of transposon insertion sites among the mutants, and an insertion of each mutant at one single site only. Following this procedure the mutants were stored in 96 well microtiter plates at -70°C for further in vivo testing in a the established chicken infection model.

It has to be mentioned that most APEC strains contain a large plasmid of about 100 kb, as does IMT5155. The presence of these large plasmids has been frequently shown to be associated with virulence of the respective strain for chickens. These plasmids also frequently encode the colicin V gene (ColV). In order to show that this virulence-associated plasmid had not been lost during mutagenesis, the plasmids from the randomly selected 18 mutants as well as from wild type IMT5155 was isolated using the method of lysis with SDS. All 19 bacterial strains analysed showed identical plasmid patterns. In addition, the ColV encoding gene was successfully amplified from all these randomly selected mutants. Overall these results suggested that the mutants had not lost the large virulence-associated plasmid. Rpresentative results are shown in Fig. 10.

a) **b)**

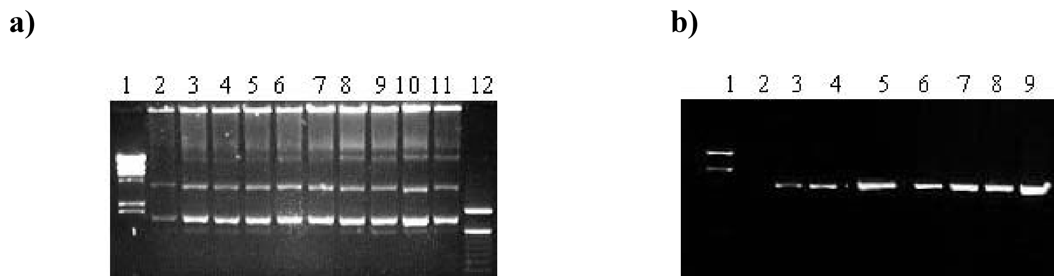


Figure 10: **a)** Electropherogramm of plasmids from IMT5155 mutants as well as the wild type. 1: 1 kb ladder; 2-10: mutants; 11: wild type IMT5155; 12: 100 bp ladder. **b)** ColV encoding genes in mutants. 1: 100 bp ladder; 3-9: randomly selected mutants.

4.3 Screening of the APEC STM bank in the chicken infection model

In summary, all necessary preliminary experiments had been performed to obtain an optimal protocol for screening in vivo attenuation of STM mutants. Critical parameters for in vivo STM approaches are selection of a highly pathogenic strain, determination of an optimal ID_{50}-value in regard to the complexity of tagged pools, and appropriate infection duration and determination of optimal time for re-isolation of the bacteria from different internal organs. A range of experimental factors including the infectious dose and the method of recovery were investigated before we could reproducibly recover the majority of individual mutants. Using intratracheal challenge doses of 10^5, 10^6, or 10^7 cfu of strain IMT5155 Nal^R, a very low percentage of animals developed typical signs of colibacteriosis. In contrast, the use of 10^9 cfu for intratracheal inoculation resulted in death of several chickens before re-isolation of bacteria could be performed. However, the inoculation dose of 10^8 cfu gave reproducible disease progression, and the bacteria could be re-isolated from about 85% of infected chickens. Irrespective of the infectious bacterial dose used for inoculation, control strain IMT5104 failed to be re-isolated from any internal organ and chicken infected with this strain had neither clinical signs of colibacillosis nor any macroscopical lesions typical of the disease as shown by necropsy.

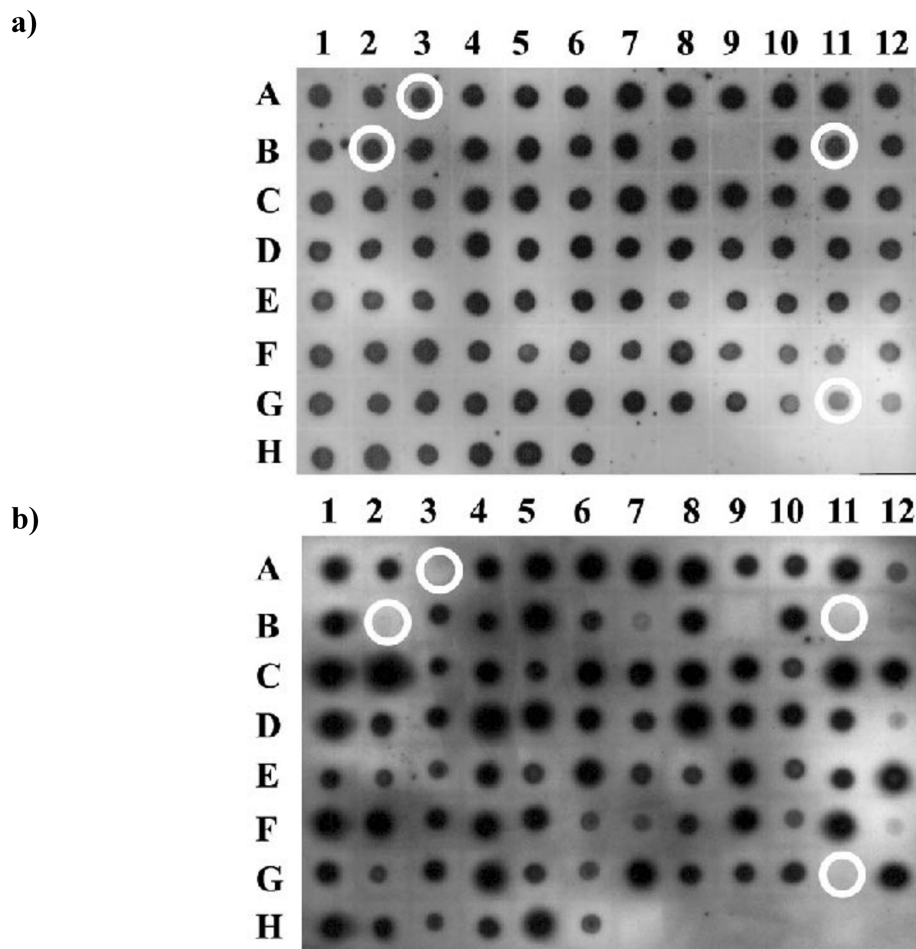


Figure 11: Illuminogram displaying example of STM results from a representative pool. As can be seen from the hybridization signals, most mutants grew as well in vivo as in vitro **(a)** Dot blot of input pool 16. **(b)** Dot blot of recovered pool 16. Hybridization signals of A3, B2, B11 and G11 were highly reduced; therefore the respective mutants were selected for further study.

In a following experiment, three randomly selected pools of 90 mutants each were used to infect four chickens each with 10^8 cfu of IMT5155 Nal^R and bacteria were recovered from the spleen after 48 and 72 h. Similar hybridization results were obtained when blots were probed with tags from bacteria recovered from at least two chickens after 48 h of infection.

In most cases three or four mutants were absent from each output pool. In contrast, unusual high numbers of mutants disappeared from the recovered pools when bacteria were re-isolated 72 h after inoculation. These findings indicated that some mutants may have simply been outgrown by others. As these results were reproducible in three trials with 12 chickens, it was decided to test pools of 90 mutants with an inoculation dose of 10^8 cfu each and to recover bacteria from spleen after 48 h of infection. In this way, a total of 1.800 mutants from 20 pools were used for screening, and a total of 65 mutants disappearing from the recovered pools were selected for further studies (Fig. 11).

4.4 Confirmation of the attenuation for STM mutants

In order to confirm attenuation of the selected mutants, a second screening of STM was performed. Mutants with loss of hybridization signal in the recovered pool compared to the input pool were reassembled into new 96-well dishes. As positive controls, DNA of mutants that hybridized to both the input and output pools were included. Four new pools containing 90 mutants were subjected to a secondary screening as described above and each pool was tested in two separate groups with four chickens to demonstrate if reproducible results could be obtained. In the second round STM screening, about 7-9 mutants from each pool could not be recovered from the output pool (Fig. 12). In total, from all 4 pools 30 mutants reproducibly showed a loss in hybridization signal from output pools.

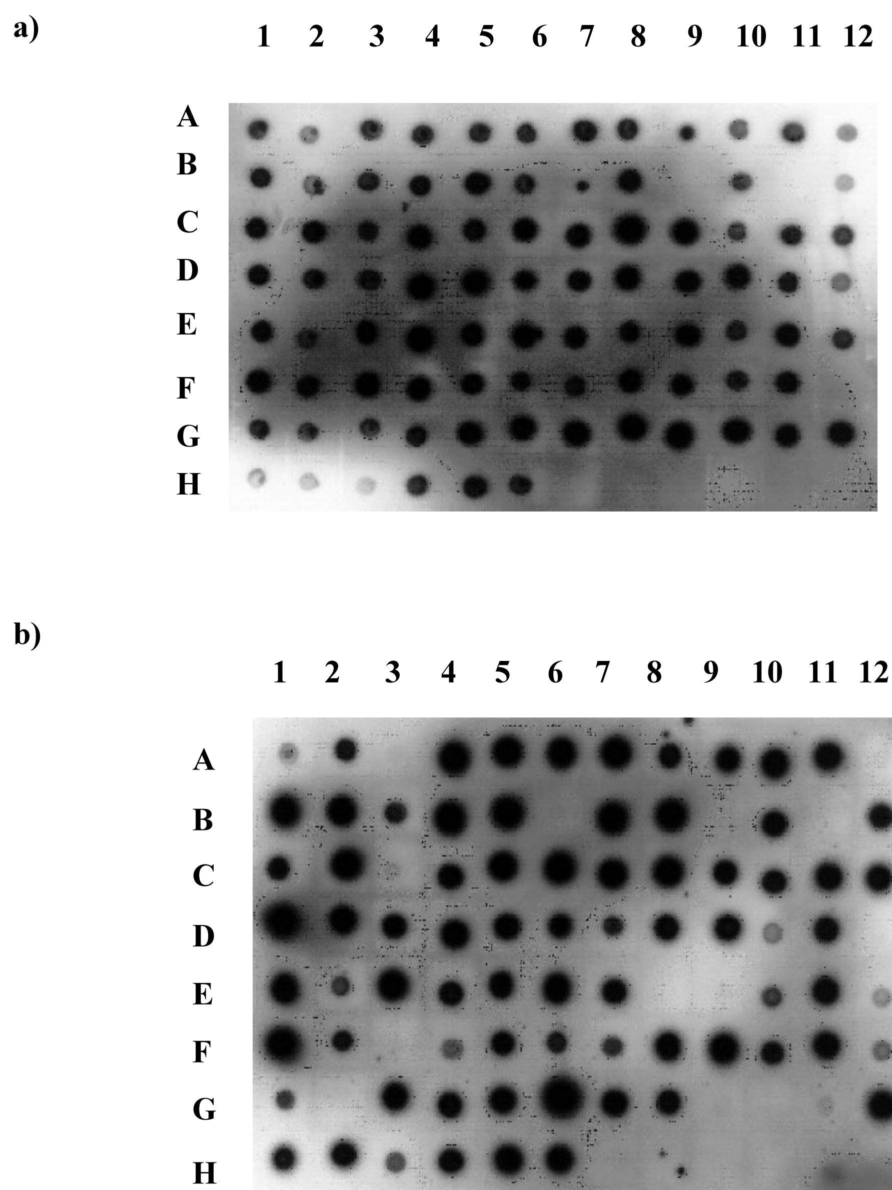


Figure 12: Example of one pool from second round of STM screening. **a)** input pool 1 of second round of STM screening. **b)** recovered pool 1 of second round of STM screening. 9 mutants (A3, A12, D12, E8, E9, F2, G2, G9 and G10) present in the input pool were absent in the recovered pool.

4.5 Determination of the insertion site of the transposon

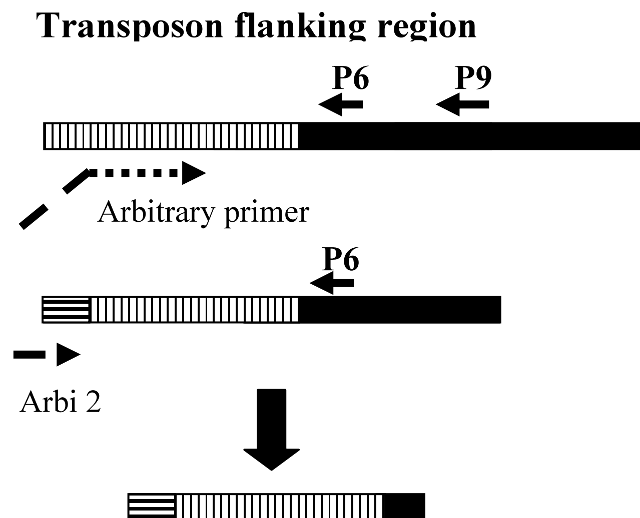
4.5.1 Arbitrary PCR

After having selected these 30 attenuated mutants, the disrupted gene loci had to be identified. Most of flanking sequences of transposon insertion could be identified by amplification via arbitrary PCR. The strategy of this arbitrary PCR is illustrated in Fig. 13a. The initial PCR amplification was done via arbitrary PCR, namely an arbitrary primer was used together with a transposon-specific primer. These primary reactions yielded numerous amplicons. The second round consisted of a nested PCR. Products from the first round PCR were used as template, utilizing primers Arbi2 which is identical to 5' sequences of the above mentioned arbitrary primer, and the transposon I terminus specific primer P6. Amplified DNA products from the second round nested PCR ranged from 150 to 750 bp and most reactions gave only one clear band when analysed via agarose gel eletrophoresis, while few reactions gave several bands including one clear main band and one or two obscure bands (Fig. 13b). The purified PCR production from the second round could be directly used for DNA sequencing. 24 insertion sites were identified by arbitrary PCR.

4.5.2 Cloning

Six insertion sites that could not be identified via arbitrary PCR had to be identified by conventional DNA cloning. The strategy chosen for cloning of the insertion sites is shown in Fig. 14. Genomic DNA of selected mutants were digested with *EcoR*I, *Sac*I, *Kpn*I, *Sal*I, *Pst*I, *Sph*I and *Sac*II which yield only one restriction site in the transposon and probed with the kanamycin gene of mini-Tn5. The 3-5 kb positive fragments were ligated with pUC19 and kanamycin resistance constructs were selected. Plasmids which were purified from kanamycin-resistant clones were used for DNA sequence analysis. The transposon insertion sites of six of the mutants were identified applying this cloning strategy. These inserted target DNAs were verified by digestion of selected restriction enzyme and analyzed via agarose gel eletrophoresis.

a)



b)

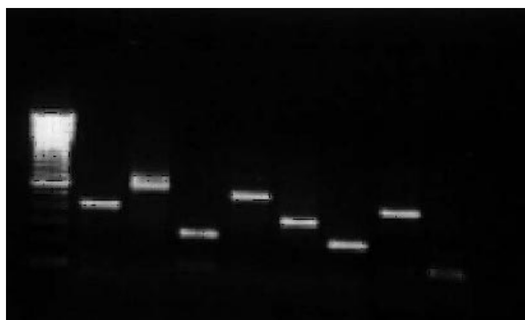
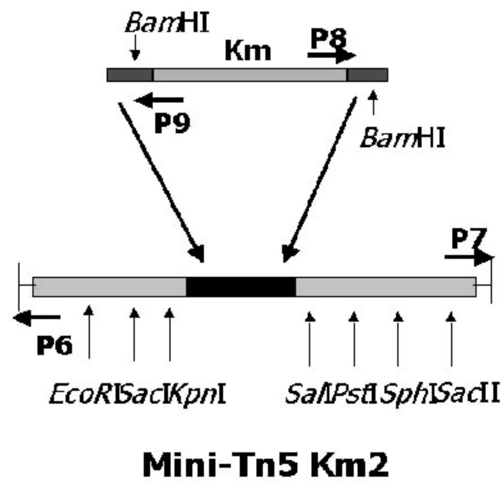


Figure 13: **a)** Strategy of the arbitrary PCR: in the first round of PCR, transposon specific primer P9 and arbitrary primer are used; in the second round nested PCR, primers are transposon I termini primer P6 and arbi2 which is identical to 5’ end of arbitrary primer used in the first round of PCR. **b)** Results of the second round of PCR. Most reactions gave only one band.

a)



b)

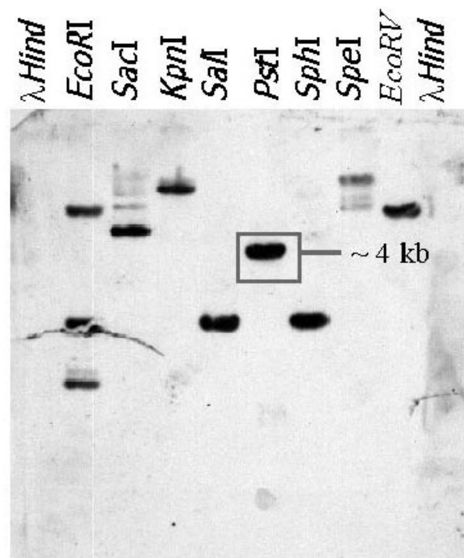


Figure 14: **a)** Genetic map of the inserted fragment of mini-Tn5. *EcoR*I, *Sac*I, *Kpn*I, *Sal*I, *Pst*I, *Sph*I and *Sac*II have only one restriction site in the transposon and have no restriction site in kanamycin resistance gene. **b)** Result example of southern blotting. *Pst*I gave a 4 kb fragment including Km gene and flanking sequence of transposon; this fragment was purified and cloned into pUC19.

4.5.3 Identification of the disrupted loci

The DNA products generated ranged from 150 to 750 bp. DNA sequencing has been finished commercially by AGOWA GmbH (Berlin, Germany) and DNA sequences were analysed by BLASTX or BLASTN, and hits with the highest score and lowest *e*-values were recorded. A total of 28 genes from the 30 identified mutants that are potentially essential for chicken *E. coli*-septicemia were identified and are now candidates for further exploratory research. Among these disrupted genes, eight encode proteins involved in biosynthesis of extracellular polysaccharides and LPS; two encode iron transporters of APEC; four encode membrane or periplasmic proteins. Some metabolic enzymes, regulators, putative proteins with unknown function and ORFs with no similarity to any known sequences were also identified (Table 3.).

Table 3. Genetic loci disrupted in mutants of APEC strain IMT5155 Nal^R identified by STM.

In vivo identified mutant		**Similar *E. coli* genes (GenBank)**			
Class	**Mutant name**	**Gene[a]**	**Accession number**	**Percentage of identity[a]**	**Putative function**
Capsule	M03A03	*kpsM*	ECU59301	97%	Polysaccharide transport
	M03E05	*kpsM*	ECU59301		Polysaccharide transport
	M18H02	*kpsS*	AAN82139	99%	Polysaccharide transport
Colanic acid	M03G02	*wcaE*	AF320069	97%	Putative glycosyltrans-ferase
LPS	M13F03	*waaW*	AAN82886	99%	LPS R1 core biosynthe-sis
	M12D10	*waaW*	AAN82886	98%	LPS R1 core biosynthe-sis
	M03D10	*waaL*	NP_756310	97%	LPS R1 core biosynthe-sis region Polymer ligase
	M00G10	none	none	0	O-antigen synthesis[b]
Iron uptake system	M04E08	*sitB*	AAN80284	98%	Iron transporter
	M11B04	*chuA*	AAN82744		Heme utilization/ trans-port protein
Membrane and periplasmic proteins	M17F12	*mppA*	ECU88242	97%	Periplasmic murein pep-tide-binding protein precursor
	M19A01	*narK*	AAN80151	93%	Nitrite extrusion
	M19B12	None	AAN80227	100%	Transport, drug/analog sensitivity
	M08E08	*sbmA*	AAN78960	96%	Putative membrane, drug/analog sensitivity, possible envelope
Metabolic	M00C03	*R3*	AAN81852	98%	Beta-cystathionase

In vivo identified mutant		**Similar *E. coli* genes (GenBank)**			
enzymes					
	M04C10	*tktA*	AAN81968	91%	Transketolase 1 enzyme, central intermediary metabolism
Regulators	M14G11	*carP*	CAA60164	91%	Pyrimidine regulation
	M18E10	*yjjQ*	NP_757290	98%	Putative regulator
Unknown	M03C05	*ykgC*	NP_752357	100%	Putative oxidoreductase
	M11E02	*malX*	NP_755283	98%	Phosphotransferase system
	M17E09	*ygcW*	AAC75816	100%	Hypothetical oxidoreductase
	M00C03	*ycjM*	NP_753684	98%	Putative polysaccharide hydrolase
	M13H03	None	AAN81851	96%	Phosphosugar isomerase
	M18E07	*ychF*	NP_753566	100%	GTP-binding proteins
	M18B06	none	AAB40738	99%	Inserted sequence
	M16D12	*yahA*	AAC73418	100%	unknown
	M00B02	*yagM*	AAC73382	96%	unknown
	M02E05	none	NP_755082	98%	unknown, hypothetical protein
	M00F07	none	none	0	unknown
	M18D02	none	none	0	unknown

a. Genetic locus with the closest match to the sequence interrupted by the transposon in each mutant.
b. As determined in this study.

4.6 In vitro and in vivo competition assay

Because STM identifies mutants that are out-competed by multiple other strains during a mixed infection, the attenuation of mutants had to be confirmed and quantified using an in vivo competition assay. In addition, the mutants could simply have growth defects, thus being unable to grow substantially in the given time. Therefore, competition assays were performed to validate the results of our STM screen and to quantify the degree of virulence attenuation of individual mutants. 19 of the 30 mutants were selected, based on their belonging to one of the 8 classes listed in Tab. 3. These 19 mutants were mixed with the parental strain at a 1:1 ratio, and either grown in vitro or inoculated into four chickens by intratracheal application.

For in vitro competion assay, the mixed bacteria were incubated in LB broth for four hours at 37 ºC and then plated on to media with or without kanamycin. Of the 19 mutants tested, the in vitro competion indices ranged from 0.17 to 2.07. For in vivo competion assay, the mixed bacteria were inoculated to 4 chickens by intratracheal application. Total bacteria were recovered from lung, liver, heart, spleen, and kidney after 48 h, and cfu were enumerated on selective medium. A competitive index (CI) was calculated as described in *Materials and Methods*. Of the 19 mutants tested, 14 (73.7%) were out-competed by the wild-type strain in all five internal organs examined, while the other five mutants (26.3%) showed reduced recovery only in certain organs. Furthermore, 15 (78.9%) of the mutants were out-competed in certain organs more than 10-fold, and of these 6 (31.6%) were out-competed more than 100-fold in certain organs, confirming the attenuation of the 19 selected mutants. The results of in vitro and in vivo competition assays are shown in Tab. 4. These results suggested that most of the 30 mutants identified by the two rounds of STM screening were reproducibly attenuated.

Table 4: Results of in vitro and in vivo competition assay between IMT5155 Nal^R and its mutants.

Mutant	Gene/ Homologue	CI[a] in vitro	CI of lung	CI of liver	CI of heart	CI of spleen	CI of kidney
M03A03	*kpsM*	1.56	3.5×10^{-1}	$<1.0\times10^{-3}$	$<1.0\times10^{-3}$	$<1.0\times10^{-3}$	1.5×10^{-1}
M18H02	*kpsS*	5.1×10^{-1}	4.2×10^{-2}	$<1.0\times10^{-3}$	$<1.0\times10^{-3}$	1.7×10^{-1}	$<1.0\times10^{-3}$
M03G02	*wcaE*	8.0×10^{-1}	$<1.0\times10^{-3}$	/[b]	2.0×10^{-4}	9.0×10^{-3}	1.2×10^{-1}
M12D10	*waaW*	7.0×10^{-1}	2.6×10^{-1}	1.0	5.1×10^{-2}	2.6×10^{-1}	1.5×10^{-1}
M03D10	*waaL*	6.0×10^{-1}	5.0×10^{-1}	2.5×10^{-1}	1.0×10^{-1}	5.1×10^{-2}	1.2×10^{-1}
M00G10	None	1.67	1.6×10^{-2}	1.0×10^{-1}	6.310^{-3}	/[b]	$2.7x10^{-1}$
M04E08	*sitB*	3.2×10^{-1}	8.3×10^{-2}	2.1×10^{-1}	4.8×10^{-2}	1.3×10^{-2}	8.8×10^{-2}
M11B04	*chuA*	9.6×10^{-1}	6.45×10^{-2}	1.8×10^{-1}	4.0×10^{-2}	4.12×10^{-2}	1.5×10^{-1}
M17F12	*mppA*	4.6×10^{-1}	6.6×10^{-1}	3.3×10^{-1}	1.0	2.0×10^{-1}	7.0×10^{-1}
M19A01	*narK*	1.40	3.8×10^{-1}	4.7×10^{-2}	3.2×10^{-2}	4.0×10^{-1}	4.8×10^{-1}
M19B12	none	6.2×10^{-1}	5.4×10^{-1}	1.2	6.1×10^{-1}	2.5×10^{-1}	1.0
M08E08	*sbmA*	6.4×10^{-1}	2.0×10^{-1}	1.4×10^{-1}	2.2×10^{-1}	2.5×10^{-1}	/[b]
M00C03	*r3*	5.2×10^{-1}	5.4×10^{-1}	6.1×10^{-1}	1.3	2.8×10^{-1}	1.1
M04C10	*tktA*	1.7×10^{-1}	5.51×10^{-1}	6.0×10^{-4}	1.7×10^{-1}	3.9×10^{-3}	9.6×10^{-2}
M21C03	*ycjM*	1.08	5.3×10^{-1}	1.7×10^{-1}	6.12×10^{-2}	4.6×10^{-1}	3.6×10^{-1}
M11E02	*malX*	1.05	3.1×10^{-2}	2.2×10^{-1}	1.6×10^{-2}	3.8×10^{-1}	2.1×10^{-2}
M13H03	none	8.0×10^{-1}	2.8×10^{-2}	3.1×10^{-1}	/[b]	2.3×10^{-1}	2.9×10^{-1}
M18E07	*ychF*	5.8×10^{-1}	3.1×10^{-2}	1.42×10^{-2}	$<1.0\times10^{-4}$	1.5×10^{-2}	1.8×10^{-2}
M18D02	none	2.07	9.0×10^{-2}	2.9×10^{-1}	2.2×10^{-1}	6.4×10^{-2}	8.5×10^{-1}

a. CI, competitive index. Values > 1 indicate mutant outcompeted wild type, values < 1 indicate wild type outcompeted mutant; mutants being out-competed up to 10-fold were evaluated as slightly, up to 100-fold as moderately and more than 100-fold as highly attenuated.
b. /, not tested

In vivo competitive indices (CIs) are the average of results gained with two to four animals.

4.7 Detection of *met*V associated sequences in IMT5155

Three newly identified loci (r2, r3 and a gene encoding a phosphosugar isomerase) in APEC IMT5155 were supposed to be localized in a pathogenicity island, as has been described in a *met*V pathogenicity island of the uropathogenic *E. coli* (UPEC) strain CFT073 (Guyer *et al.*, 1998). In order to identify a similar pathogenicity island, we tried to amplify the left and right junction of the IMT5155 *met*V island, the *hly*, *pap* and *prrA-modD-yc73-fepC* gene locus as well as insertion sequences *r14* and *r15*. The amplification of *prrA-modD-yc73-fepC* gene locus, right junction of *f447* locus and insertion sequences *r14* and *r15* were successful, but the trial to detect both the *hly* and *pap* gene locus failed. However, the link *r3*, *r2*, *r1* and *f447* could be confirmed using PCR (Tab. 5).

Table 5: Detection of *met*V island-associated genes in *E. coli* strain IMT5155

Primer	Sequence (5´- 3´)	Presence of gene
PapG-F	TTTGCGAGTGGAGTGTATTT	-
PapG-R	TACCTAACCCAACCGAAAAT	
PapC-F	TGATATCACGCAGTCAGTAGC	-
PapC-R	CCGGCCATATTCACATAA	
R14-15-F	GCCAGTGACACATACTGAGAGC	+
R14-15-R	CAGATGTACAGTGGCGCG	
R2 plus R3-F	GCTGTCAGAATATTTCGCTCG	+
R2 plus R3-R	AGTCCTGTCACGCTGAACG	
R1 pus R2-F	AGCCTTTCTGTTTTGAGCAT	+
R1 plus R2-R	TCGCTACTATTGATTCTTGC	
R1 plus f447-F	CCGCAAGAATCAATAGTAGC	+
R1 plus f447-R	CTGGCGAGAAGGGGATAATG	

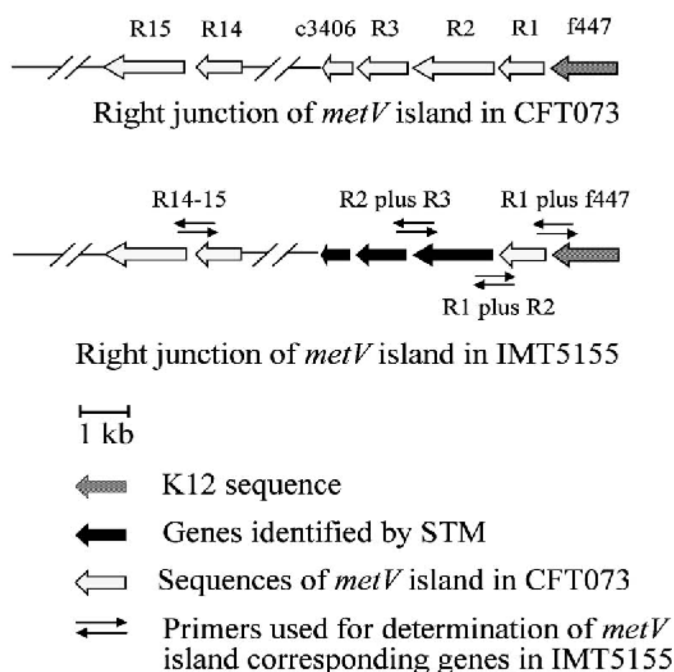


Figure15: Schematic representation of the *met*V island-associated sequences of APEC strain IMT5155**.** Genes *r2* (syn. *malX*), *r3* and c3406 (phosphosugar isomerase) were identified in APEC strain IMT5155 by STM. Their links with *r1* and *f447* were confirmed by PCR with primers outlined in Tab. 2.

4.8 LPS expression analysis

In order to confirm deficiency for LPS synthesis, LPS of four mutants showing disrupted genes involved in LPS synthesis and of the wild type strain IMT5155 was isolated and then subjected to SDS-PAGE and silver staining analysis. Compared to the parental strain, all four mutants showed lower LPS production. As expected, M13F03 (*waa*W) and M12D10 (*waa*W) both showed band differences in the LPS core region when compared to wild type strain. Mutant M03D10 (*waa*L) had alterations in both O-antigen and core region while M00G10 (no similarity to any known gene) demonstrated different banding patterns in the O-antigen region, only (Fig. 16).

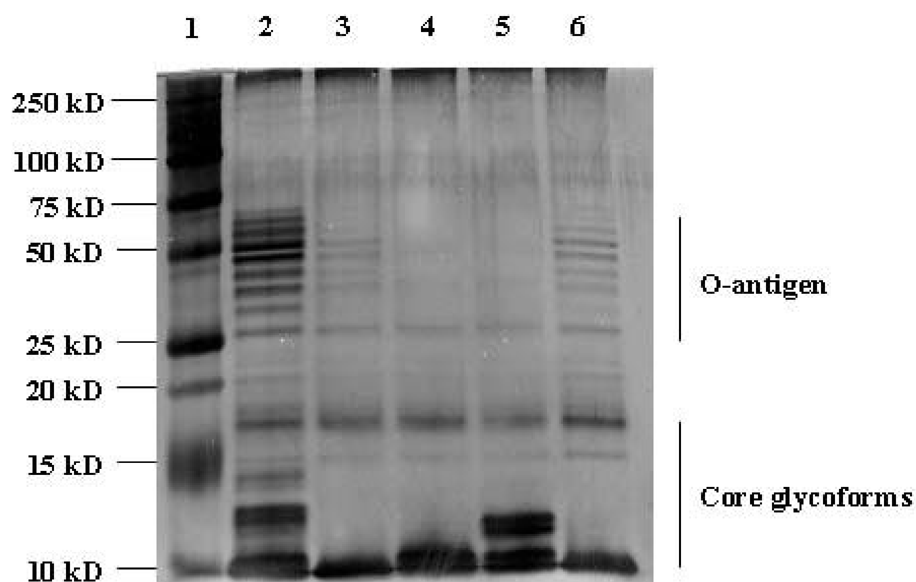


Figure 16: SDS-PAGE analysis of LPS from mutants and parental strain. Lane 1: ladder; lane 2, wild type; lane 3, *waaW* mutant (M13F03); lane 4, *waaL* mutant; lane 5, mutant M00G10; lane 6, *waaW* mutant (M12D10). The position of the repeating subunits of the O-antigen and the core region are indicated. All mutants yielded lower LPS production compared to the wild type, and *waaW* mutants had altered LPS core structures; *waaL* mutant showed difference in both O-antigen and the core region and mutant M00G10 had altered O-antigen region.