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Characterization of Large Plasmids Encoding Resistance to Toxic Heavy Metals in *Salmonella abortus equi*

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***Salmonella abortus equi* vaccine strains were found to be resistant to high levels of toxic heavy metals—arsenic, chromium, cadmium, and mercury. The two strains 157 and 158 were resistant to ampicillin also. Curing of these strains resulted in loss of one or more resistance marker indicating plasmid borne resistance. Plasmid profile of strain 157 showed presence of three plasmids of 85, 54, and 0.1 Kb, whereas 158 strain showed presence of 85 Kb and 2 Kb plasmids. Plasmids were isolated from strain 157 and introduced into *E. coli* DH5α with a transformation efficiency of 2×10^3 transformants/μg DNA. Interestingly the transformants were resistant to antibiotics, heavy metals (As, Cr, Cd, Hg) and was also able to utilize citrate, a trait specific to Salmonella species. We report and establish for the first time the transferable large plasmids encoding resistance to various heavy metals, antibiotics and biochemical nature of *S. abortus equi*.** © 2000 Academic Press

***Key Words: Salmonella abortus equi;* heavy metal resistance; curing; transformation; total DNA; citrate utilization.**

Salmonella abortus equi common in tropical condition is infectious in equines of all ages. The subtle and variable symptoms are metritis followed by abortions in mares and arthritis and orchitis in stallion. In foals, the acute form of the disease shows the symptom of septicaemia, pericarditis, peritonitis and broncho pneumonia (Verma *et al.*, 1992). Despite the importance of vaccine development against this deadly disease of equines, until now no work in its molecular biology has been presented, resulting in inability to genetically manipulate this species.

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An important issue regarding the construction of genetic vectors is the availability and characterization of plasmids. The presence of plasmid(s) in several serotypes of salmonella have been reported and the plasmids have been shown to mediate for certain characters viz. virulence, serum, antibiotic resistance, etc. (Barrow *et al.*, 1987; Jones *et al.*, 1991). However, the information on extrachromosomal DNA is not available on *S. abortus equi*. The vaccine producing strain of *S. abortus equi*, against which vaccine is produced by conventional method at Indian Veterinary Research Institute, Izatnagar, India has been used for present studies.

Environmental pollution by heavy metals as a result of fossil fuel burning and industrial discharges is increasing. In this case of water bodies, the continued influx of pollution load is aggravated in summers when the water evaporates increasing metal content. During this process, many bacteria acquire metal tolerance and the plasmid expression can lead to antibiotic resistance also. This may lead to new disease patterns and difficulties in management of infections. Bacterial resistance to heavy metals in polluted environment is wide spread. For each toxic heavy metal resistance mechanism is quite specific (Chu *et al.*, 1992). It appears that in nature most, but not all, toxic metal ion resistance determinants occur on plasmids. While most resistance systems function by energy dependent efflux of toxic ions, some involve enzymatic transformations (Silver *et al.*, 1992; Gupta *et al.*, 1999). In methicillin-resistant S. aureus, mercury resistance is determined by a chromosomal 6.4 Kb Bgl II fragment that shows strong DNA sequence homology with the 6.4 Kb Bgl II fragment from plasmid pI258 (and related plasmids) that determines the same range of resistance (Witte *et al.*, 1986). It is clear that some of these heavy metal resistance determinants have moved from plasmid to chromosome (or in the reverse direction). This makes plasmid encoding heavy metal resistance an important aspect of environmental research. These plasmids can

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be the source of resistance genes for cloning purpose; which have potential use in biotechnology such as the manufacture of biosensors and bioremediation processes (Collard *et al.*, 1994). In view of the above we report the screening of *S. abortus equi* strains for the presence of extra chromosomal DNA, the finding of large hard-to-isolate plasmids in the majority of the strains and the studies performed with two of these strains.

MATERIALS AND METHODS

Two vaccine strains, of *Salmonella abortus equi* were kindly provided by National Salmonella Centre and Biological Products Division, IVRI, Izatnagar, India. The above strains were subjected to a battery of tests for identification and characterization viz. growth curve. H and O Antigen, biochemical tests, antibiotic sensitivity, heavy metal resistance, etc. Details of antibiotic sensitivity and heavy metal resistance are given in Table 1. *Escherichia coli* DH5α (Hanahan, 1983) was used as the host strain for transformation. *S. gallinarum* E402 was molecular mass reference plasmids of molecular weight 85 Kb and 2 Kb. Both *S. abortus equi* as well as *E. coli* strains were grown at 37°C in LB supplemented with appropriate concentration of antibiotic.

Resistance to antibiotics and toxic heavy metals. The disc diffusion technique of Bauer and Kirby *et al.* (1966) was followed for antimicrobial sensitivity test. To determine the minimum inhibitory concentration, agar dilution method of Cruickshank (1968) was adopted. Resistance to heavy metals viz. lead, mercury, calcium, chromium, nickel, arsenic was determined by the methods of Luli *et al.* (1983). The metal salts used were mercury iodide, cadmium acetate, potassium dichromate, nickel chloride, arsenic oxide, and lead acetate (Qualigenes).

Extraction, purification, and manipulation of plasmid DNA. To extract plasmid DNA from *S. abortus equi* strains, the cells were lysed with alkali and the plasmid DNA was purified by precipitation with polyethylene glycol according to Maniatis *et al.* (1989) and using elutip-d starter kit (Pharmacia Co.). The *S. abortus equi* plasmids isolated and described in this work were named PAE followed by the identification code of the host strain. The plasmids were purified from low melting point agarose for restriction enzyme digestion as described by the methods of Maniatis (1989). The eluted plasmids were digested with restriction enzyme's EcoRI and Hind III as described by Maniatis *et al.* (1989). The molecular weight of plasmids from different strains were determined by the method described by Maniatis (1989). The *S. gallinarum* E402 with plasmids of 85 kb and 2 kb was kept as reference molecular weight marker. Curing of different plasmid determinant traits by acridine orange at 37°C was conducted as described by Poppe *et al.* (1987). Transformation of *E. coli* DH5α and cured *S. abortus equi* strain 157 A.O. was achieved following the procedure described by Maniatis (1989). The transformants along with *S. abortus equi* strain 157, *E. coli* DH5α and cured salmonella strain were subjected to serological, biochemical tests, antibiotic sensitivity, and plasmid profile. The transformation efficiency was determined which is the ratio of transformants per μg of DNA.

$$\text{Transformation efficiency} = \text{No. of transformants}/\mu\text{g DNA.}$$

Total DNA from *S. abortus equi* strain was isolated essentially as described by Cutting and Vander Horn (1990). Unsheared genomic DNA was prepared and digested with EcoRI, Hind III, restriction endonuclease as described by Maniatis *et al.* (1989).

Gel electrophoresis. Conventional DNA gel electrophoresis was carried out as described by Meyers *et al.* (1976) in 0.7% agarose gels and 0.5 × (0.09 M Tris-borate and 0.002 M EDTA) at 60 V (3.0 V cm^{-1}) for 3 h. Gels were strained with 0.5 μg of ethidium bromide per milliliter and photographed through a red filter.

TABLE 1

Antibiotic Susceptibility and Resistance for Heavy Metals

		Zone of inhibition	
	Disc potency	*S. abortus equi* 157	*S. abortus equi* 158
Antibiotic			
Penicillin	10 IU	R	R
Oxacillin	5 mcg	R	R
Erythromycin	15 mcg	R	R
Bacitracin	10 IU	R	R
Vancomycin	30 mcg	R	R
Lincomycin	10 mcg	R	R
Tetracycline	30 mcg	S	S
Kanamycin	30 mcg	S	S
Streptomycin	10 mcg	R	S
Chloramphenicol	30 mcg	S	S
Gentamycin	10 mcg	R	S
Ampicilin	10 mcg	R	R
Heavy metals	mg/ml		
Cadmium	100	R	R
Chromium	50	R	R
Nickel	100	R	R
Arsenic	100	R	R
Mercury	5	R	R
Lead	6400	R	R

RESULTS

Antimicrobial Susceptibility and Resistance for Heavy Metals

Drug resistance pattern of both strains of *S. abortus equi* showed resistance to penicillin G, oxacillin, lincomycin, vancomycin, bacitracin and erythromycin. Where as one strain was resistant to streptomycin and both strain were resistant to and ampicillin (Table 1). Resistance to heavy metals with highest rate was observed with lead, cadmium, chromium, arsenic, nickel and mercury (Table 1).

Screening of S. abortus equi Strains for Plasmid DNA

Plasmid DNA from *S. abortus equi* strains were detected in large scale preparation (3000 ml. of culture medium containing approximately 10^9 cells ml^{-1}). Attempts to improve the yield by addition of chloramphenicol (170 mg ml^{-1}) to the culture did not have much effect. Both the strains showed presence of one large plasmid, but in strains 157 three and two bands respectively DNA profile was revealed in agarose gel (Figs. 1 and 2). The bands of both the strains migrated to a distance similar to that for the 85 kb and 2 kb plasmid of reference strain of *S. gallinarum* E402, suggesting the presence of similar high molecular mass

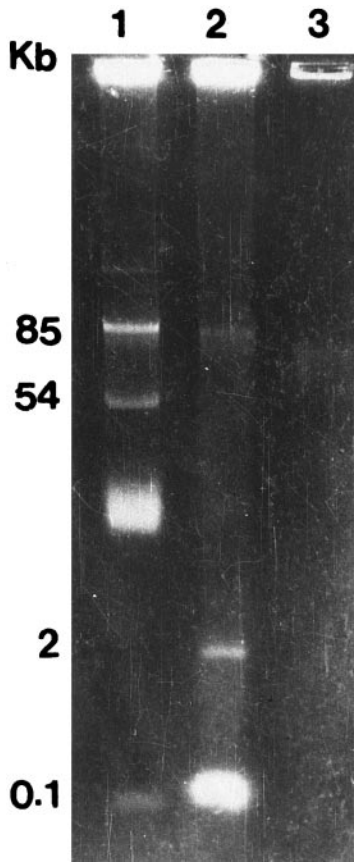

FIG. 1. Agarose gel electrophoresis of plasmid isolated from *S. abortus equi* strain 157 (Lane 1), reference plasmid of 85 Kb and 2 Kb from *S. gallinarum* E401 in (Lane 2).

plasmids. In addition of 85 kb plasmid, strain 157 had one large plasmid of 54 kb and one small plasmid of 0.1 kb.

Restriction Analysis of Plasmid

The presence of more than one plasmid in strain 157 required elution and purification of plasmids from low melting point agarose gel (Sigma Co.) and by costor microfuge tube with Durapore membrane, Ultrafree-MC unit. The third plasmid of 0.1 kb was hard to be eluted because of its being a low copy number and small size plasmid. The plasmids PAE1 (85 kb) was digested with Hindi III and PAE2 (54 kb) with EcoRI giving 7 and 8 DNA fragments respectively (Fig. 2).

Plasmid Curing

The *S. abortus equi* strain 157 and 158 resistant to the antibiotic ampicillin and heavy metals arsenic, cadmium, chromium, and mercury were converted to sensitive (Table 2). In both strains arsenic and cadmium were cured at a frequency of 100%. In strain 157 mercury resistance was cured at 100% whereas in strain

FIG. 2. Agarose gel electrophoresis of plasmid's restriction enzyme digestion of strain 157 (Lane 10). Lambda DNA Hind III cut (Lane 9). *S. abortus equi* strain 157 plasmid's of 85, 54, and 0.1 Kb (Lane 8). Strain 157, 85 Kb plasmid eluted from gel and cut with Hind III (7 fragments) (Lane 7). Strain 157, 54 Kb plasmid eluted and cut with EcoRI (8 fragments) (Lane 6-1). Acridine orange cured colonies, showing absence of plasmids.

158 mercury resistance was cured at 96% with acridine orange of 100 μ/ml. Chromium resistance was cured at a frequency of 92% and 94%. There was no curing for nickel and lead resistance. One very interesting character was discovered on biochemical tests, the citrate utilizing property of *S. abortus equi* was lost on curing, thus showing presence of this particular gene on plasmid. The results of this experiment indicates presence of genes responsible for both the above mentioned traits on plasmid as the elimination of plasmid, evident by plasmid profile of cured strain (Fig. 2), rendered them sensitive to antibiotic and heavy metals.

Transformation

The plasmid profile of transformant *E. coli* DH5α and *S. abortus equi* strain 157 A.O. (cured) showed presence of two DNA bands namely plasmid pAE1 and pAE2 (Fig. 3). Biochemical test of transformant colonies and cured strain highlighted that the citrate uti-

TABLE 2

Curing of Resistance in *S. abortus equi*

	Percentage cured	
Traits	157	158
Antibiotic		
Ampicillin	69	100
Heavy metals		
Cadmium	100	100
Chromium	92	94
Nickel	16	Nil
Arsenic	100	100
Mercury	100	96
Lead	20	16

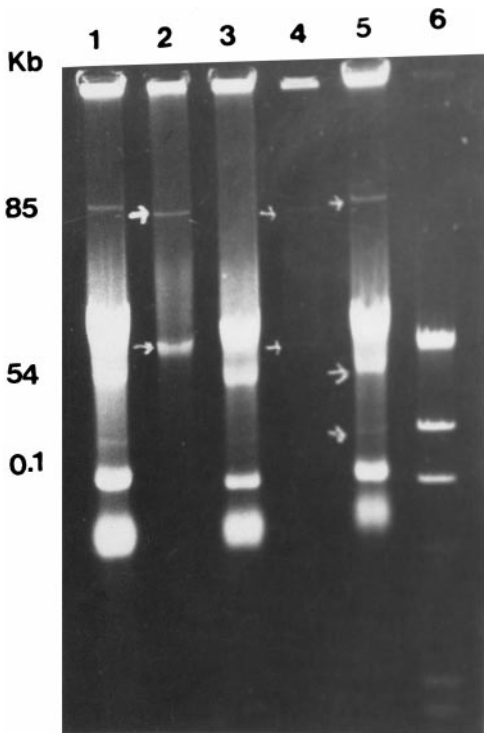

FIG. 3. Agarose gel electrophoresis of plasmids isolated from transformants (Lane 1). *S. abortus equi* 157, (Lanes 2 and 4) transformant *E. coli* DH5α (Lanes 3 and 5). Acridine orange cured *Salmonella abortus equi* strain transformed with plasmid DNA (Lane 6). Lambda DNA cut with Hind III.

lization trait lost in curing was regained on transformation indicating presence of citrate utilization gene on plasmid along with antibiotic ampicillin resistance and metal resistance genes for mercury, arsenic, cadmium and chromium (Table 3).

Total DNA from strain 157 and 158 of *S. abortus equi* was digested with EcoRI, Hind III (Fig. 4) which cut the genomic DNA in a ladder like pattern.

DISCUSSION

Strain 157 of *S. abortus equi* resistant to ampicillin was chosen for detailed study because of wide spread use of ampicillin in veterinary practice and its importance in creating selective pressure in organisms. Combined expression of resistance to antibiotics and of resistance to heavy metals by genes present on the plasmid suggests the contamination with heavy metals, by exerting selective pressure on bacteria. The elevated rate of resistance to metals reflected an adaptive response to the presence of toxic elements in different environments such as water, sewage, rivers, estuaries. The heavy metal resistance genes are often found on plasmid and transposons (Chu *et al.*, 1992; Silver *et al.*, 1992). The mercury resistance development in bacteria is due to plasmid mediated proteins responsible for reduction and protein binding and removal of organic ligands (Silver *et al.*, 1992). The presence of multiple transporters in a bacterial cell for a single ion, the presence of ion transporters with multiple specificities, and the occurrence of both influx and efflux transporters for the same ion all add to the complexity of transport and required the cell to carefully regulate transporters (Silver, S., 1978). The regulation of gene expression of anion and cation transport system is gaining attention for the desire to

TABLE 3

Result of Transformation Experiments

				Transformants	
Character	*S. abortus equi* 157	*E-coli* DH5α	*S. abortus equi* cured	*S. abortus equi* DH5α	*S. abortus equi* cured
Antibiotic resistance					
Ampicillin	R	18 (S)	18 (S)	R	R
Kanamycin	R	21 (S)	19 (S)	R	R
Penicillin G	R	10 (S)	R	R	R
Oxacillin	R	R	R	R	R
Vancomycin	R	R	R	R	R
Erythromycin	R	R	R	R	R
Rifampicin	22 (S)	R	16 (S)	R	16 (S)
Amikacin	15 (S)	22 (S)	23 (S)	18 (S)	32 (S)
Heavy metal resistance					
Arsenic	R	S	S	R	R
Cadmium	R	S	S	R	R
Cromium	R	S	S	R	R
Mercury	R	S	S	R	R
Nickel	R	S	S	R	R
Lead	R	S	S	S	S
Cirtrate utilization	+	−	−	−	+

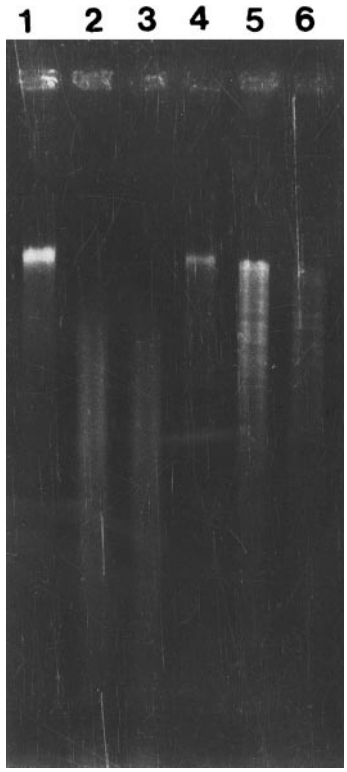

FIG. 4. Restriction enzyme digestion of Total DNA of *S. abortus equi* (Lane 1). Uncut total DNA of strain 157, (Lane 2) DNA of strain 157 cut with Eco RI (Lane 3). DNA of strain 157 cut with Hind III, (Lane 4). Total DNA of strain 158 (Lane 5). DNA of strain 158 cut with Eco RI, (Lane 6). DNA of strain 158 cut with Hind III.

control transport for use of micro organisms in biotechnology and bioremediation. Heavy metal pollution of waste water is a problem for which bioremediation by microorganisms may be a natural solution, since control of transport of metal ions might augment the ability of micro organisms to extract deleterious ions (Silver *et al.,* 1992). The genes governing metal resistance are found on plasmids and ion transports are chromosomally governed (Desjardins *et al.,* 1988; Gupta *et al.,* 1999). Association between resistance to antibiotics and heavy metals have been reported by several workers (Ernesto *et al.,* 1993; Dhakephalkar and Chapade, 1994; Ramteke, 1997). Under environmental conditions of metal stress, such metal and antibiotic resistant population will adopt faster by the spread of R-factors than by mutation and natural selection thus leading to a very rapid increase in their numbers (Bhattacharjee *et al.,* 1988). Multiple heavy metal resistance determinants, namely the Cd-Co-Zn genes (CZC), the Co-Ni-Cr genes (Cnr, Chr) and the Hg (mer) have been isolated from plasmids (Collard *et al.,* 1994; Gupta *et al.,* 1999). The plasmid profile of *S. abortus equi* showed presence of a common plasmid of 85 Kb in all the strains. This can be very helpful in identification of strains serotyping (Threlfall *et al.,* 1990).

In *Salmonella gallinarum* large plasmid were cured at varying frequency indicating the involvement of plasmid in virulence (Poppe *et al.,* 1987; Barrow *et al.,* 1987). Similar report was presented by Chakrabarty *et al.* (1984) and Attfiled *et al.* (1985), for loss of plasmid linked drug resistance after treatment with indodeoxy uridine. Here we found that curing with acridine orange resulted in loss of ampicillin resistance, As, Cr, Cd, and Hg resistance and ability to utilize citrate. Verma *et al.* (1992) had reported curing of antibiotic resistance in *S. abortus equi* but this is the first instance to report curing of heavy metal and citrate utilizing traits in *S. abortus equi.*

The location of resistance genes for antibiotics, metal resistance and citrate utilization on plasmid of *S. abortus equi* was confirmed by transformation of *E. coli* DH5α cells and cured strain of *S. abortus equi* with the plasmid DNA of *S. abortus equi,* whereby the transformants showed all the characteristics of the *S. abortus equi* strain. On transconjugation (not included in this paper) resistance for heavy metal lead and nickel were also transferred to *E. coli* K-12, which was not evident on transformation with *E. coli* DH5α. This clearly indicates that the genes for lead and nickel resistance are chromosome mediated hence it was transferred to *E. coli* K-12 on transconjugation. This report tally with studies of Silver S. (1978).

The results indicated that *S. abortus equi* plasmid replicated in *E. coli* DH5α and plasmid possessed genetic information necessary for the expression of antibiotic resistance, metal resistance and citrate utilization. Correlation of 36 Md plasmid in *S. enteritidis* for virulence in mice was confirmed by transformation by Suzuki *et al.* (1989). The *E. coli* DH5α transformants showed all other characteristics of *S. abortus equi* except citrate utilization trait which was not expressed in *E. coli.* Whereas the cured *S. abortus equi* strain was able to repossess the citrate utilizing gene. It indicates that the gene responsible for expression of citrate utilization is partially present on chromosome. And for the expression of citrate utilizing traits in the organism presence of both the chromosomal as well as plasmid gene is necessary.

CONCLUSION

The screening of two *S. abortus equi* strain for extrachromosomal DNA revealed the presence of plasmid of 85 kb in one strain and presence of three plasmids of 85, 54, and 0.1 kb in other strain (157). Curing with acridine orange rendered the strain devoid of plasmid as well as traits for antibiotic resistance, heavy metal resistance and citrate utilization. The transformation of *E. coli* DH5α cells and cured *S. abortus equi* 157 A.O. rendered them resistant to antibiotic ampicillin and heavy metals, arsenic, chromium, cadmium and mercury. The cured strain 157 A.O. has once again ability

to utilize citrate. Thus we conclude that *Salmonella abortus equi* strain harbour large antibiotic and heavy metal resistant plasmids. The role of plasmid was confirmed by transformation to *E. coli* DH5α and plasmid cured strain of *S. abortus equi*. To our knowledge this is the first attempt which shows transfer of gene responsible for heavy metal resistance and citrate utilization in *E. coli* DH5α and cured *Salmonella abortus equi*.

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