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PCR Based Detection Of Genes Encoding Virulence Determinants In *S.aureus* Strains Isolated From Bovine Milk

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ABSTRACT

A total of 84 ***S.aureus*** strains were isolated from subclinically mastitic bovine milk samples using traditional methods. Twenty eight *S.aureus* isolates were subjected to PCR for detection of coagulase gene, X region of protein A (spA) gene and panton valentine leukocidin (PVL) gene. All the isolates (100%) were positive for coagulase gene that showed no gene polymorphism, while 26 (92,8%) isolates were positive for spA gene that showed gene polymorphism with different sized amplicons. .Only 3 (10.7%) isolates were positive for PVL gene. Multiplex PCR used for detection of enterotoxins genes (SEA, SEB, SEC and SED) in the same 28 isolates where 8 isolates (28.5%) were positive to SEA gene, one (3.5%) was positive for SEC gene , one (3.5%) was positive for both SEA and SEC genes and all isolates (100%) were negative for SEB gene . Hence 35.7% of these isolates possess enterotoxins genes.

INTRODUCTION

S. aureus is recognized worldwide as a frequent cause of subclinical intramammary infections in dairy cows *(1)*. One of *S. aureus* virulence factor is coagulase, an exoenzyme which clots plasma by conformational activation of prothrombin *(2)*.

Protein A is located in the cell wall of *S.aureus* hindering the phagocytosis process by capturing antibodies .The gene encoding protein A (*spa*) is composed of some functionally distinct regions: Fc binding region, X region and at C terminus, a sequence required for cell wall attachment *(3)*.

Recent studies suggest that staphylococcal enterotoxins (SETs) act as virulence factors in cattle *(4)*. Among other effects, these superantigens induce in this species is the production of interleukin 4 and 10 which activate TH2 cells leading to reduced clearance of microbial pathogens *(5)*.

One of the virulence components of *S.aureus* is leukotoxin. Leukocidins were shown to be cytotoxic for erythrocytes and leukocytes including bovine neutrophils and macrophages *(6)*. These toxins are considered as virulence factors in bovine mastitis and may play an important role in the development of this disease *(7)*.

The objective of this study was to determine different exotoxins and virulence factors genes of *S.aureus* isolated from milk of lactating animals by using polymerase chain reaction including coagulase, protein A, PVL and enterotoxins genes.

MATERIAL AND METHODS

Sampling

A total of 480 individual milk samples were collected from apparently normal 120 lactating animals (50 local breed buffaloes and 70 Friesian cows) from Ismailia Governorate, Egypt and were examined for subclinical mastitis.

California mastitis test (CMT)

Milk samples were subjected to CMT (screening test) for the detection of subclinical mastitis. Milk samples with CMT scores ±, +, ++ and +++ were prepared for bacteriological examination.

Isolation and identification of *S.aureus*

Milk samples were aerobically incubated for 24 hours at 37 °C to achieve potential bacterial growth. The incubated milk sample was centrifuged 3000 rpm for 5 min , then the supernatant (cream layer) was discarded and sediments were streaked onto nutrient agar, blood agar and Mannitol salt agar plates .All plates were incubated at 37°C for 24-48 hours and examined daily for bacterial growth.

Bacterial colonies were identified morphologically using Gram's stain as well as biochemically *(8)*.

Genetic detection of different virulence factors genes of *S.aureus* isolates by using polymerase chain reaction:

1-Extraction of DNA from *S.aureus* isolates by boiling method *(9)*

2-Polymerase chain reaction: DNA samples were tested [in 50 µl. Reaction volume in a 0.2 ml PCR tube , containing PCR buffer] (50 mM Kcl , 10 mM tris - Hcl , 1mM $Mgcl_2$) each dNTPS (Deoxy nucleotide Triphosphate) 200 uM each (dATP , dGTP, dCTP and dTTP) , [Two primer pairs each at 50 picomol / reaction] and 0.5 of taq DNA polymerase . Thermal cycling in a programmable heating block (Coy vorporation, Grasslake, Michan, USA) was done.

3-Screening of PCR products: Ten µl of amplified PCR product was analyzed by electrophoresis on a 2% agarose gel stained with 0.5 µg of ethedium bromide / ml. Electrophoresis was carried out in 1X TAE buffer at 80 volt for 1 hour. Gels were visualized under UV transilluminator (UVP, UK) and photographed.

Table 1. list of primers used for PCR assay

Primer	Primer Sequence.	Annealing temperature	PCR program	Refrences
Coa1	ATA GAG ATG CTG GTA CAG G	58°c	1	(10)
Coa 2	GCT TCC GAT TGT TCG ATG C			
SEA-3*b*	CCT TTG GAA ACG GTT AAA ACG	55°c	2	(11)
SEA-4*b*	TCT GAA CCT TCC CAT CAA AAA C			
SEB-1*c*	TCG CAT CAA ACT GAC AAA CG	55°c		
SEB-4*b*	GCA GGT ACT CTA TAA GTG CCT GC			
SEC-3*b*	CTC AAG AAC TAG ACA TAA AAG CTA GG	55°c		
SEC-4*b*	TCA AAA TCG GAT TAA CAT TAT CC			
SED-3b	CTA GTT TGG TAA TAT CTC CTT TAA ACG	55°c		
SED-4*b*	TTA ATG CTA TAT CTT ATA GGG TAA ACA TC			
spa-III	CAA GCA CCA AAA GAG GAA	60°c	3	(12)
spa-IV	CAC CAG GTT TAA CGA CAT			
luk-PV-1	ATCATTAGGTAAAATGTCTGGACAT GATCCA	66°c	4	(13)
luk-PV-2	GCATCAACTGTATTGGATAGCAAAA GC			

(1): 39 times (94 °C for 1 min.; 58 °C for 1 min. ; 72 °C for 1 min)

(2): 30 times (95 °C for 1 min.; 55 °C for 1 min. ; 72 °C for 2min.)

(3): 30 times (94 °C for 1 min. ; 60 °C for 1 min. ; 72 °C for 1 min.)

(4): 34 times (94 °C for 30 sec. ; 66 °C for 30 sec.; 72 °C for 1 min.30 sec.)

RESULTS

Table 2. Incidence of subclinical mastitis in examined quarter milk samples of buffaloes and cows according to California mastitis test

Animal species	No. of examined animals	No. of milk samples	No. of - -ve samples	No. of +ve samples	% of +ve samples
buffaloes	50	200	112	88	44
cows	70	280	134	146	52.14
Total	120	480	246	234	48.75

Table 3. percentage of *S.aureus* subclinical bovine mastitis

Total Sample examined	No .of affected samples	No. of *S.aureus* isolates	Percentage of *S.aureus*
480	234	84	35.9

Regarding the virulence factors and exotoxins genes of *S.aureus* isolates, 28 *S.aureus* isolates were subjected to PCR for detection of coagulase gene, X region of protein A (spA) gene and panton valentine leukocidin (PVL) gene. All the isolates (100%) were positive for Coagulase gene (with specific molecular weight 600bp.) that showed no gene polymorphism (Photo 1), while 26 isolates (92,8%) were positive for spA gene that showed gene polymorphism with different sized amplicons (140 bp, 270 bp and 290 bp) (Photo 2). Only 3 isolates (10.7%) were positive for PVL gene with specific molecular weight 433bp (Photo 3).

Multiplex PCR protocol used for detection of enterotoxins genes (SEA, SEB, SEC and SED) where 8 isolates (28.5%) were +ve to SEA gene (127bp), one (3.5%) was +ve for SEC gene (271bp) , one (3.5%) was + ve for both SEA and SEC genes and all isolates (100%) were negative for SEB gene. hence 35.7% of these isolates possesses enterotoxins genes (Photo 4 and Photo 5)

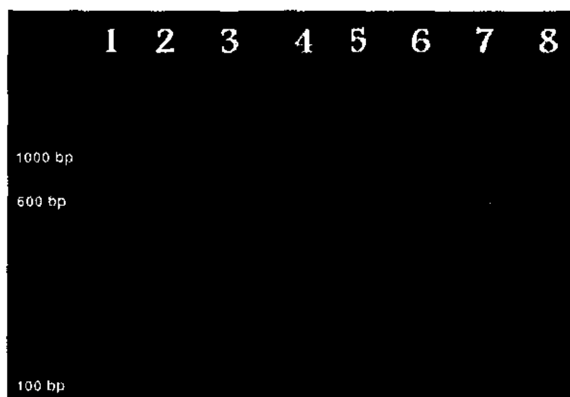

Photo 1. Electrophoretic pattern of coagulase PCR assay

lane 1 (100 bp DNA ladder), lane 2 control coagulase positive *S.aureus* strain, lane 3 control negative and lane 4-8 showed coagulase positive isolated *S.aureus* (600 bp).

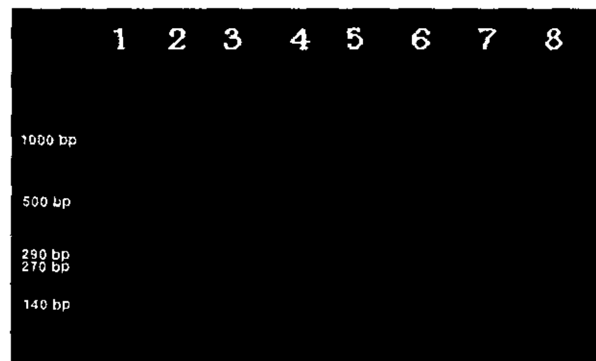

Photo 3. Electrophoretic pattern of protein (A) PCR assay

Lanes 1 and 8: (100 bp DNA ladder), Lanes 2 and 7: +ve isolates with double specific bands (at 270bp ans 290 bp.) , Lanes 3 and 6 : +ve isolates with specific band (140 bp), Lane 4 : +ve isolate with specific band (270 bp) , Lane 5: +ve isolate with specific band (290 bp).

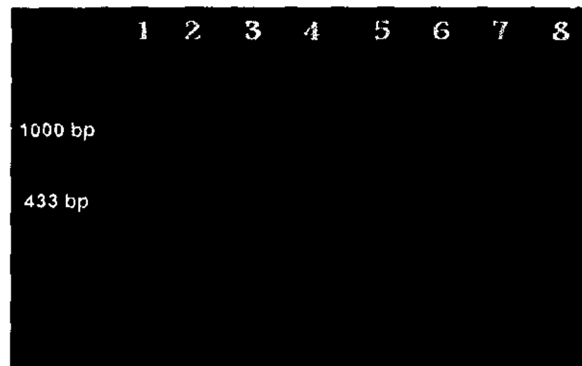

Photo 3. Electrophoretic pattern of (PVL) PCR assay

Lanes 1: (100 bp DNA ladder); Lanes 2, 4 and 7: +ve isolates for PVL gene with specific band (433 bp); Lanes 3, 5 and 6: -ve isolates for PVL gene.

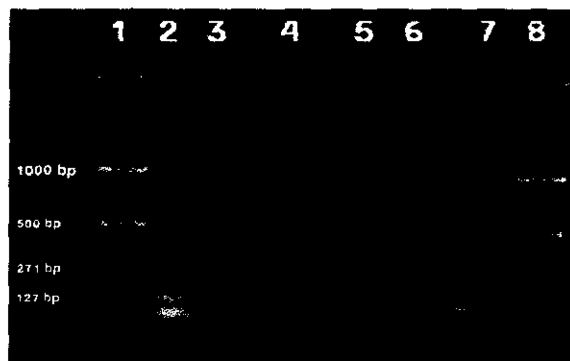

Photo 4. Electrophoretic pattern of Enterotoxines PCR assay

Lanes 1 and 8 : (100 bp DNA ladder); Lanes 2, 3, 6 and 7 : +ve for SEA (127bp); Lane 4: +ve SEC (271 bp); Lane 5: -ve isolate for entertoxines.

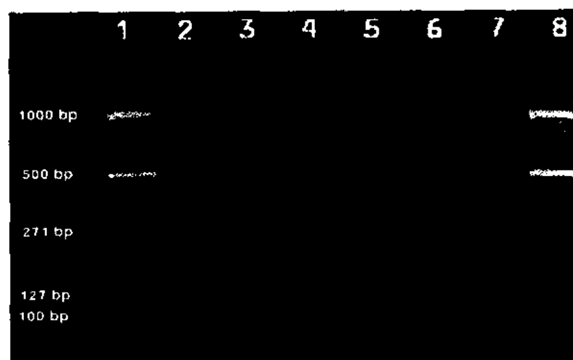

Photo 5. Electrophoretic pattern of Enterotoxines PCR assay
Lanes 1 and 8 : (100 bp DNA ladder); Lanes 3, 4, 6 and 7 : +ve for SEA (127bp); Lane 2: +ve for SEC (271bp); Lane 5:+ve for both SEA (127 bp) and SEC (271 bp).

DISSCUSSION

As shown in Table 2 the prevalence of subclinical mastitis was 48.75 %.The higher prevalence of bovine subclinical mastitis has been previously reported by *(14 , 15)*. The increased prevalence of subclinical mastitis among dairy animal may be attributed mainly to poor hygiene practices, inadequate housing and bedding, malfunctioning milking machines, improper milking procedures and inadequate treatment methods. Treatment failures of subclinical mastitis are common and may be due to delayed treatment, poor selection of drugs and dose level, stopping treatment too soon, resistance of invasive organisms or deep seated infections that are protected by scar tissues *(16)*.

Results illustrated in Table 3 revealed that the percentage of *S.aureus* recovered from subclincally mastitic quarter milk samples was (35.9%).The recorded rate of *S.aureus* subclinical mastitis in the present study has been demonstrated in Elhiopia *(15)* and in Nerweigian *(17)* herds. Although lower rate of *S.aureus* isolation (6.5%) in Estonian diary herd was reported *(18)*. *S.aureus* is responsible for approximately 30% to 40% of all mastitis cases. *S. aureus* can gain access to milk either by direct excretion from udders with clinical or subclinical staphylococcal mastitis or by contamination from the environment during handling and processing of raw milk *(19)*.

In this study PCR protocol was used for amplification and detection of coagulase gene, Photo 1 illustrated the positive amplification of 600 bp fragment of Coa gene. These results are agreed with those obtained *(12)*. The amplification of coagulase gene resulted in a single amplicon, indicating no size polymorphisms of this gene *(20)*. However, coagulase gene amplification in other study *(19)* resulted in different amplicons, indicating coagulase gene size polymorphism. At present, no information is available about the sequence variation of these strains.

PCR protocol used for amplification and detection of X region of protein A (spA gene) of *S.aureus* isolates were carried to confirm the virulence of these isolates as the presence of protein A is an index of virulence. Different sized amplicons were found (140 bp, 270 bp and 290 bp) as shown in Photo 2. Protein A displayed gene polymorphisms and allowed a genotypic characterization of the bacteria. The repetitive region X of the *spa* gene includes a variable number of 24-bp repeats. The number and sequence of individual repeats may differ among strains. The number of repeats has been related to the dissemination potential of *S. aureus*. Strains with more than seven repeats in the X region tended to be epidemic, while the presence of seven or less repeats was indicative of a non-epidemic methicillin-resistant *S. aureus* strain *(21)*.

PCR protocol used for amplification and detection of panton valentine leukocidin (PVL) gene of *S.aureus* isolates. Twenty eight isolates were subjected to PCR for detection of panton

valentine leukocidin (PVL) gene. Only 3 (10.7%) isolates were +ve(433 bp) as shown in Photo 3. The presence of PVL gene in *S. aureus* of bovine origin was agreed with the results obtained *Eiji (22)* but disagrees with those obtained *Ikawaty et al. (23).* PVL is the most active *S. aureus* leukotoxin on bovine neutrophils, PVL has greater leukotoxic activity against bovine polymorphnuclear cells than other bicomponent toxins thus, PVL may contribute to resistance to the attack of bovine polymorphnuclear cells. The clarification of these phenomena may aid in elucidating mechanisms of *S. aureus* infection and pathogencity against each host *(24).*

In the present study, multiplex PCR was used for detection of *S.aureus* enterotoxins as virulence factors of *S.aureus,* 28 isolates were subjected to multiplex PCR for detection of genes (SEA, SEB, SEC and SED), where eight (28.5%) isolates were +ve to SEA gene , one (3.5%) was +ve for SEC gene , one (3.5%) was + ve for both SEA and SEC genes and all isolates (100%) were negative for SEB gene, hence 35.7% of these isolates possesses enterotoxins genes as shown in Photos 4 and 5, with regard to the gene encoding enterotoxins the most frequent enterotoxin is SEA followed by SEC that agreed with results obtained from raw and pasteurized milk isolates strains *(25).* In spite of the great discrepancy in data concerning the prevalence of enterotoxigenic *S. aureus* isolates found in the literature, which is attributable to different types of foods and biovars, SEA is the most frequently observed enterotoxin in enterotoxigenic strains of *S. aureus (26).*

In conclusion, owing to specificity, rapidity and ease of use the conventional and multiplex PCR methods described in this study can be profitably applied for rapid characterization of *S.aureus* strains isolated from milk and for epidemiological studies. These procedures were specially developed to fit into the daily work pattern of a routine clinical laboratory, since genotypic detection of virulence genes and the presence of toxin genes is becoming an important component of the diagnostic inventory of such laboratories.

REFERENCES

1. ***Marrack P and Kappler J (1990):*** The staphylococcal enterotoxins and their relatives. Science 248:705–711.

2. ***Panizzi P , Friedrich R , Fuentes-Prior P , Bode W and Bock P E (2004):*** The staphylocoagulase family of zymogen activator and adhesion proteins. Cell Mol. Life Sci. 61, 2793–2798.

3. ***Foster T J and McDevitt D (1994):*** Surface-associated proteins of ***S aureus***: their possible roles in virulence.FEMS Microbiol.Lett.**118, 199-205.**

4. ***Chang B S , Bohach G A , Lee S U , Davis W C , Fox L K ,Ferens Seo K S , Koo H C , Kwon N H and Park Y H (2005):*** Immunosuppression by T regulatory cells in cows infected with *Staphylococcal superantigen*. J. Vet. Sci. 6, 247–250.

5. ***Burton J L and Erskine R J (2003):*** Immunity and mastitis. Some new ideas for an old disease. Vet. Clin. North Am. Food Anim. Pract. 19, 1-45.

6. ***Loeffler D A , Schat K A and Norcross N L (1986):*** Use of 51Cr release to measure the cytotoxic effects of staphylococcal leukocidin and toxin neutralization on bovine leukocytes.J.Clin.Microbiol.23, 416–420.

7. ***Younis A , Krifucks O , Fleminger G , Heller E D , Gollop N , Saran A and Leitner G (2005):*** *S. aureus* leucocidin, a virulence factor in bovine mastitis. J. Dairy Res. 72, 188–194.

8. ***Quinn P J , Carter M E , Makrkey B K and Carter G R (1994):*** Clinical veterinary microbiology mosby year book Europ Limited, Lynton House, London,.109-126.

9. ***Van Eys G M , Gravekamp C , Gerritsen M , Quint W , Cornlissen S J and Terpstra W (1989):*** Detection of *leptospira* in urine by PCR.j.Clinc.Microbiol.27:2258-2262.

10. ***Hookey J V, Richardson J F and Cookson B D (1998):*** Molecular typing of *S. aureus* based on PCR restriction. Vet. Microbiol. ,80:131–138.

11.***Becker K , Roth R and Peters G (1998):*** Rapid and specific detection of toxigenic *S. aureus*: Use of Two Multiplex PCR Enzyme Immunoassays for Amplification and Hybridization of Staphylococcal Enterotoxin Genes Exfoliative Toxin Genes and Toxic Shock Syndrome Toxin 1 Gene J. Clinc. Microbiol. , 36(9): 2548–2553.

12.***Akineden O , Annemu C , Hassan A A , Lammler C , Wolter W and Zschock M (2001):*** Toxin Genes and Other Characteristics of ***S. aureus*** Isolates from Milk of Cows with Mastitis. Clinical and Diagnostic Lab. Immunology , 8(5):959-964.

13.***Kristin K , Bonnstetter D J , Wolter F C ,Tenover , Linda K M and Richard V G (2007):*** Rapid Multiplex PCR Assay for Identification of USA300 Community-Associated Methicillin-Resistant *S. aureus* Isolates. J. Clin. Microbiol. , 45(1): 141–146.

14.***Chowdhury M , Saha G R , Chowdhury J and Das S K (2002):*** Subclinical mastitis in crossbred cows. Journal of interacademicia 6 (special) 712-715 .

15.***Dego O K and Tareke F (2003):*** Bovine mastitis in selected areas of southern Ethiopia.Trop Anim Health Prod., 35 (3):197-205.

16.***Philipot W N (1984):*** Mastitis and management . 2nd ed. Babson Bros. Comp. 21005 York Road USA.

17.***Whist A C , Osteras O and Sølverød L (2007):*** *S. aureus* and *S. dysgalactiae* in Norwegian herds after introduction of selective dry cow therapy and teat dipping. J. Dairy Res. ,74(1):1-8.

18.***Haltia L , Honkanen B T , Spiridonova I, Olkonen A and Myllys V (2006):*** A study of bovine mastitis milking procedures and management practices on 25 Estonian dairy herds . Acta Vet Scand. ,22 ,48(1):22-31.

19.***Scherrer D, Corti S , Muehlherr J E , Zweife C and Stephan R (2004):*** Phenotypic and genotypic characteristics of *S. aureus* isolates from raw bulk-tank milk samples of goats and sheep. Veterinary Microbiology ,101 :101–107.

20.***Stephan R , Annemuellerb C , Hassanb A A and Laemmler C (2001):*** Characterization of enterotoxigenic *S. aureus* strains isolated from bovine mastitis in north-east Switzerland .Veterinary Microbiology , 78 :373-382.

21.***Frenay H M , Theelen J P , Schouls L M , Vandenbroucke-Grauls C M , Verhoef J , van Leeuwen W J and Mooi F R (1994):*** Discrimination of epidemic and nonepidemic methicillin-resistant *S aureus* 44 strains on the basis of protein A gene polymorphism. J Clin Microbiol , 32 ,846-847.

22.***Eiji H , Ken K , Hideki K , Ikuo U , Kiyoshi T and Masashi E (2010):*** Genetic Variation among *S aureus* Strains from Bovine Milk and Their Relevance to ethicillin-esistant Isolates from humans. J. Clin. Microbiol. (under press). www. pubmed. com.

23.***Ikawaty R , Brouwer E C , Van D E , Mevius J V and Fluit A C (2010):*** Virulence factors of genotyped bovine mastitis ***S.aureus*** isolates in The Netherlands. Int. J. Dairy Sci. , 5: 60-70.

24.***Barrio M B , Rainard P and Prevost G.(2006):*** LukM/LukF-PV is the most active *S. aureus* leukotoxin on bovine neutrophils. Microbes Infect. , 8:2068–2074.

25.***Rall V L M , Vieira F P , Rall R , Vieitis R L , Fernandes J , Candeias J M G , Cardoso K F G and Arau´jo J P (2008):*** PCR detection of staphylococcal enterotoxin genes in *S. aureus* strains isolated from raw and pasteurized milk Veterinary Microbiology , 132 : 408–413.

26.***Normanno G , Firinu A , Virgilio S , Mula G , Dambrosio A , Poggiu A , Decastelli L , Mioni R , E , Salinetti A P, La Salandra G , Sias S , Parisi A , Quaglia, N C and Celano G V (2005)***: Coagulase-positive staphylococci and *S. aureus* in foods products marketed in Italy. Food Microbiol. , 98: 73–79.

الملخص العربى

استخدام اختبار البلمرة المتسلسل في تحديد الجينات الخاصة بضراوة الميكروب العنقودي الذهبي المعزول من البان الماشية

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تم عزل و تصنيف (٨٤) عترة من الميكروب المكور العنقودى الذهبى من البان الماشية الحلابة المصابة بالتهاب الضرع السريرى..وقد تم اجراء اختبار البلمرة المتسلسل (ل ٢٨ من المعزولات) وذلك لتحديد تواجد جينات (Coagulase, . protein A and PVL). هذا وقد وجد ان كل المعزولات كانت موجبة لجين coagulase الذي لم يظهر أى تنوع فى الحجم الجينى بعكس جين proteinA الذى أظهر تنوع فى الحجم الجينى .وكان هناك ثلاث معزولات فقط تحمل جين PVL.وأيضا قد تم اجراء اختبار البلمرة المتسلسل المتعدد على نفس المعزولات وذلك لتحديد وجود جينات السموم المعويه (SEA, SEB, SEC and SED). وقد أظهرت النتائج ان ٨ معزولات تحمل جين SEAوواحده تحمل جين SEC وواحدة تحمل كلا من SEA /SECوكانت جميع المعزولات لا تحمل جين SEB .و بذلك تكون نسبة العترات التى تحمل جينات السموم المعويه ٣٥,٧% .