AN ASSOCIATION OF MEMBRANE DAMAGING TOXINS FROM COAGULASE NEGATIVE STAPHYLOCOCCUS AND CHRONIC OROFACIAL MUSCLE PAIN.

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Abstract
Forty-six patients presenting with chronic orofacial muscle pain and eight age and sex matched control subjects, selected on the basis of the absence of musculoskeletal symptoms, were investigated for the carriage prevalence and exotoxin production of coagulase negative staphylococcus (CNS). There was a higher prevalence and multiple carriage of four or more strains of CNS in patients with chronic muscle pain than with control subjects (23 vs 9 isolates /10 subjects). Two of the 103 CNS isolates from chronic muscle pain patients and none from the control CNS strains produced TSST-1 suggesting pyrogenic toxins do not contribute to the aetiology of chronic muscle pain. There was a higher prevalence of delta (δ)-haemolysin (41 of 114, P<0.04) and 'horse'-haemolysin (56 of 114, P<0.005) production by CNS strains recovered from chronic muscle pain patients compared with those from control subjects. None of the control subjects were colonised with CNS that produced significant amounts of either δ- or 'horse'-haemolysin. By comparison, 35 of 44 chronic muscle pain patients were colonised with CNS producing significant 'horse'-haemolysin (P<0.00001); 37 with δ-haemolysin (P<0.00001) and 33 with both δ- and horse- haemolysin (P<0.0001). This study suggests that membrane damaging toxins, like δ- and 'horse'-haemolysin, may have a role in the aetiology of chronic orofacial muscle pain.

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Introduction

Chronic orofacial muscle pain (craniomandibular) disorders form part of the group of chronic musculoskeletal pain conditions that may intermittently affect up to 75% of the population with severe forms occurring in 5-10% of subjects (1). The aetiology of these conditions is unknown. Patients with chronic muscle pain report symptoms predominantly in an axial skeletal distribution with increased sternal and limb pain in more severe forms (2). These clinical signs and symptoms have been strongly associated with recurrent infective disease processes in a previous study (3). These patients reported an increased prevalence of recurrent upper respiratory and genitourinary infective events at or preceding onset but with no reported evidence for increased prevalence of viral infections such as measles, mumps, and glandular fever (4). Examination of genitourinary microbial flora revealed a higher carriage rate of coagulase negative staphylococci (CNS) in patients with chronic muscle pain compared with sex matched controls suggesting an association between CNS carriage and chronic muscle pain (5).

The role of CNS in disease processes has not been well defined. Gemmel and Schumacher-Perdreau (6) demonstrated that CNS can produce as many as eight exotoxins that could contribute to virulence. Of these exotoxins, a membrane damaging toxin resembling *Staphylococcus aureus* delta (δ)-toxin showed the greatest biological activity. Cytotoxicity studies with human embryonic lung fibroblasts demonstrated that this CNS membrane damaging toxin caused preferential release of cell constituents and cellular swelling (7). Another membrane damaging exotoxin, alpha (α)- toxin, had been shown to permeabilize smooth muscle cells (8,9) suggesting that staphylococcal membrane damaging exotoxins may interfere with the excitation-coupling mechanisms of smooth muscle.

The purpose of this study was to investigate the carriage prevalence of staphylococcus in chronic orofacial muscle pain patients and symptom free control subjects and to assesses the ability of these organisms, to produce membrane damaging and other exotoxins.

Materials and Methods

Patient and control subject selection

Forty-six patients presenting for chronic orofacial muscle pain management were recruited. Patients were selected to comply with the group 1 (Muscle disorders), category 1a (myofascial pain) of the orofacial research diagnostic criteria (10). Criteria for inclusion were as follows: 1) symptoms of orofacial muscle pain; 2) a positive response on a

visual analogue pain scale (VAS) of average pain intensity in the 2 weeks prior to consultation; 3) the presence of palpable muscle pain in the reported pain areas; 4) that the pain was present on greater than 50% of days during the three months immediately preceding consultation; and 5) positive palpation of head, neck and shoulder muscles (11,12) in the sites reported to be painful were positive. Criteria for exclusion were as follows: 1) Pain was not associated with the teeth, temporomandibular joint (TMJ) clicking or arthritis and crepitation, sinusitis, salivary glands, nerve or vessel pathology; 2) reporting of pain without associated palpable muscle tenderness.

In order to select an ideal control group without any musculoskeletal symptoms potential control subjects were screened on the basis of: 1) a nil response to questions from the Hopkins Symptom Check List-90-Revised (SCL-90-R) questions Q12-chest pain, Q27-low back pain, Q42-muscle soreness, Q56-body weakness and Q58-heavy feelings in limbs (13); 2) no evidence of palpable muscle pain; 3) no response to the VAS of average pain intensity in the previous 2 weeks: and 4) had not sort professional advice or treatment for chronic muscle pain in the previous 12 months. Acute pain associated with trauma during the preceding 12 months was not a criterion for exclusion. Consequently, eight age and sex matched control subjects were recruited who complied with the highly restrictive symptom definition. Each patient and control subject provided informed consent and was interviewed and assessed by one clinician (NMcG).

Specimen collection and processing

Nose, throat, and for the female participants, vagina swabs were collected and transported in Stuart's medium (Transystem, Interpath Services P/L), and cultured on Columbia horse blood agar (Oxoid). After incubation for 24 hours at 35^0C, all cultures were examined under a stereomicroscope (Zeiss, Germany) for different morphological types of staphylococcal isolates. A single colony representing a morphological type was streaked onto horse blood agar and incubated at 35^0C for a further 24 hours. Experience from a previous study (4) demonstrated the necessity to sample a minimum of three colonies of a morphological type from each site to establish adequate representation. Cultures with a positive Gram and catalase reaction were then identified according to their biochemical characteristics.

Identification

The identification of staphylococcal isolates was performed by the methods of Kloos and Bannerman (14), Herbert et al.(15), and Kloos and Schleiffer (16). Carbohydrates in purple agar base (Difco) and other test reagents were prepared, sterilised and added to 8 wells x 12 strips of a microtitre plate. Each strip of 8 wells was prepared as follows : 1) 5µL of a sterile 20% carbohydrate solution was placed in the first six wells, 2) 100µL each of urease and nitrate solution was transferred to the remaining two wells, and 3) 95µL of sterile purple agar was added to the first six wells with the carbohydrate solution and mixed gently. Each well of each row contained a different carbohydrate/reagent solution. A solution of staphylococcal culture in saline prepared to a turbidity of a 0.5 MacFarland was used as the inoculum. The six carbohydrates tested in each strip were: lactose, maltose, trehalose, mannitol, sucrose and mannose. Only strains that were susceptible to furazolidone (100µg) and resistant to bacitracin (0.04U) were included in this study (15). A tube coagulase with citrated rabbit plasma was used to differentiate the *Staphylococcus aureus* group from the CNS group. A positive coagulase reaction with human plasma and a positive ornithine decarboxylase activity were used for the confirmation of *Staphylococcus lugdunensis* (17). Resistance to the antibiotics novobiocin (5µg) and polymyxin B (300U) were included in the differentiation of *Staphylococcus saprophyticus* and *Staphylococcus epidermidis* respectively from other related species (15). Similarly bacitracin (10U) was included to help differentiate *Staphylococcus haemolyticus* from other novobiocin susceptible staphylococci (15). All equivocal identifications were confirmed with an API Staph kit (Bio Merieus, France).

Staphylococcal supernatant

Staphylococcal strains were cultured in 5mL of brain heart infusion broth (Oxoid, England) at 35^0C for a minimum of 18 hours. Cultures were centrifuged at 25,000g at 4^0C for 20 min and each supernatant was filtered through a 0.22µm membrane filter (Millipore, Bedford, USA). Each strain was tested for toxic shock syndrome toxin (TSST)-1, enterotoxins A-D (SEA-SED) and haemolysin production.

Detection of TSST-1 and SEA-SED

Staphylococcal TSST-1 and enterotoxin types A, B, C, and D were detected by the reverse passive latex agglutination kits (SET, and TSST-RPLA Oxoid, England), used as microslide agglutination tests described by Murrell (18). Each test sample was accompanied by positive and negative known controls. Agglutination was observed with phase contrast microscopy.

Assay for haemolytic activity

Rabbit, sheep, horse and human type 'O' red blood cells were washed three times in 1mM

MgPBS (0.5M KH_2PO_4, 0.5MKH_2PO_4, 1mM $MgSO_4$) and a final 1%(v/v) of each of the four red cell types was prepared. An equal volume of the sterilised staphylococcal supernatant was added to each of the four 1% (v/v) red cell pellets. The suspensions were mixed and incubated at 35^0C for 30 min. The 'hot-cold' effect of beta (β)-haemolysin on sheep red cells was demonstrated by a further incubation at 4^0C for 1 hr. The 'heat stable' effect of delta (δ)-, haemolysin was demonstrated by heating the staphylococcal supernatant at 80^0C for 15 minutes prior to incubation with human red cells (19). The 'heat stable' effect of 'horse'-haemolysin was also investigated. After incubation, all suspensions were centrifuged for 5 min and the absorbency of each supernatant determined at 541 nm. A sample substituting deionised water for MgPBS of each of the four red cell types was measured at neat to represent 100% haemolysis and at 1:2 and 1:4 dilutions. Haemolytic activities of samples were calculated from a standard curve.

Definition of positive (significant) haemolysis

Positive haemolysis for each of the four haemolysins is defined as a value greater or equal to two standard deviations (SD) above the mean haemolysis produced by staphylococcal strains from control subjects, thus representing a high value seen in only 2.5% of a normally distributed population. The mean haemolysis percentage and SD for the four haemolysins produced by staphylococcal isolates from control subjects were: for α-haemolysin, 2.6% and 7.8% respectively ; β-haemolysin , 10.1% and 19.7%; δ-haemolysin, 0.7% and 1.2%; and 'horse'-haemolysin, 2.5% and 2.2%. Positive (significant) haemolysis for each of the four haemolysins was therefore defined as: >18.1% for α-haemolysin; >49.5% for β-haemolysin; >3.1% for δ-haemolysin; and >6.9% for 'horse'-haemolysin. Negative haemolysis for each of the four haemolysins included all values that were less than two SD above the mean haemolysis of control staphylococcal strains.

Statistical analysis

Prevalence differences between the two clinical groups were compared using Chi square test with Yates' correction when appropriate. The Fisher's exact probability test was used when detection frequency was less than one. Staphylococcal isolates with similar biochemical characteristics recovered at different sites but from the same individual were counted as one organism type. Isolates with similar biochemical characteristics recovered from the same individual but producing different membrane damaging or pyrogenic toxins were defined as different organisms.

Results

Patient and control subjects characteristics

The mean age of the 44 muscle pain patients and the eight control subjects was similar with 37.6±16.5 years (range 16-57 yr) for the control subjects and 38.3±10.5 years (range 20-56 yr) for the muscle pain patients. The sex ratio (male:female) for the muscle pain group was 1:3, compared to 1:2.7 for the control subjects.

Relationship between coagulase positive and negative staphylococcus.

A total of 125 staphylococcal isolates were recovered from the study; 11 isolates were from control subjects, and 114 from patients with chronic muscle pain (Table 1). Fifteen of 125 isolates were *Staph.aureus* and 110 were CNS. Of the 15 *Staph.aureus* recovered, 4 were isolated from control subjects and 11 from chronic muscle pain patients. This prevalence of *Staph.aureus* per 10 subjects was higher in controls than in patients with chronic muscle pain (5 *vs* 3 isolates respectively). By contrast there was a higher prevalence of CNS in chronic muscle pain patients (23 isolates/10 patients) compared with control subjects (9 isolates/10 subjects, $P<0.01$) suggesting that patients with chronic orofacial muscle pain were predisposed to multiple colonisation of CNS. The 110 CNS strains were further examined according to their biochemical characteristics (Table 1). Six CNS species were identified in the study where *Staph.epidermidis, Staph.haemolyticus,* and *Staph.xylosis* were represented in each of the two clinical groups whereas *Staph.warneri*, *Staph.hominis* and *Staph.lugdunensis* were only recovered in the muscle pain patients. This difference in species distribution was not significant suggesting that there were no dominance of a specific staphylococcal species.

Multiple staphylococcal carriage

Control subjects and muscle pain patients were screened for the incidence of multiple staphylococcal carriage. Of the 44 chronic muscle pain patients examined, 38(86.4%, $P<0.008$) were each colonised with two or more CNS strains, and 23 (52.3%, $P<0.006$) were each colonised with three or more staphylococcal strains (Table 2). Twelve of these 23 muscle pain patients with multiple carriage were also found colonised with four or more different strains of CNS. By comparison three (37.5%) control subjects were colonised with two CNS strains and the remaining five (62.5%) subjects with one or less strains of the organisms.

Staphylococcal pyrogenic toxins

The possibility that staphylococcal isolates from patients with chronic muscle pain may produce pyrogenic toxins was investigated. All staphylococcal strains recovered from the two clinical groups were screened for the production of enterotoxins A, B, C, and D and toxic shock syndrome toxin (TSST-1). The four *Staph.aureus* isolates from control subjects produced five pyrogenic toxins (Table 3). Conversely only four of the 11 *Staph.aureus* from muscle pain patients produced pyrogenic toxins. Five of the 103 CNS isolates from muscle pain patients and none from the seven control CNS strains produced pyrogenic toxins. From these data no significant association was found between the production of TSST-1 and enterotoxins amongst the muscle pain and control isolates suggesting pyrogenic toxins do not significantly contribute to the aetiology of chronic muscle pain.

Staphylococcal membrane damaging toxins

All staphylococcal isolates from the study were examined for positive and negative haemolysin activity as defined in the Materials and Methods (Table 4). There was a higher prevalence of haemolysis to red cells by isolates from chronic muscle pain patients (60 of 114, 53%) compared with those from the control subjects (2 of 11, 18%) (P<0.03). The incidence of *Staph.aureus* isolates from chronic muscle pain patients with positive haemolysin (5 of 11, 46%) was higher than, but not significantly different to that from control subjects (1 of 4, 25%). By contrast, 64 (62%) of the 114 CNS strains from chronic muscle pain patients but only one of seven (14%) CNS strains from control subjects were haemolysin positive (P<0.04), suggesting that CNS from chronic muscle pain patients were associated with increased production of membrane damaging toxins.

Staphylococcal α, β, δ, and 'horse'- haemolysins

In this study, four staphylococcal membrane damaging toxins (α, β, δ, and 'horse'-haemolysins) were examined to determine if they were responsible for the observed increased prevalence of haemolysin production among staphylococcal isolates from the muscle pain group. One of the 11 control isolates and 10 (8.7%) of 114 muscle-pain-associated isolates produced α-haemolysins causing significant haemolysis to rabbit red cells (Table 5). Similarly only one of 11 control isolates and one (<1%) of 114 muscle-pain-associated isolates produced β-haemolysins resulting in significant haemolysis to sheep red cells. These data indicate that α- and β-haemolysins were not associated with the observed increased prevalence of haemolysin production among staphylococcal isolates. When strains were examined for δ- haemolysins, 41 (36 %) of the muscle-pain-associated isolates produced significant haemolysis to human red cells compared to none of the control isolates (P<0.04). There was also a highly significant difference in the haemolytic activity to horse red cells between the two clinical groups where 56 of 114 (49.1%) muscle-pain-associated CNS isolates produced significant haemolysis to horse red cells compared with none of the control strains (P<0.005). Of the 114 muscle pain strains examined, the incidence of positive δ-haemolysis was found to be similar in strains of *Staph.warneri* (66.7%), *Staph.haemolyticus* (54.5%), *Staph.lugdunensis* (50.0%), and *Staph.hominis* (43.8%), which were significantly higher than those observed in *Staph.aureus* (18.2%, P<0.05) and *Staph.epidermidis* (15.6%, P<0.05) (Table 6). Similarly the incidence of positive 'horse'-haemolysis was comparable with all the CNS but lower with *Staph.aureus.* None of the eight control subjects were colonised with staphylococci that produced positive δ and/or 'horse'- haemolysins. By contrast, 39 (88.6%) of the 44 chronic orofacial muscle pain patients were colonised with at least one CNS producing either positive δ- or 'horse'-haemolysis: 35 (79.5%) were colonised with CNS strains producing positive 'horse'-haemolysin (P<0.00001), 37 (84.1%) with positive δ-haemolysis (P<0.00001), and 33 (75%) with both δ-and 'horse'- haemolysis (P<0.0001)

Discussion

Comparison of patients with chronic orofacial muscle pain and symptom free control subjects showed marked differences in the carriage of CNS and their production of membrane damaging toxins. The high prevalence of multiple carriage of CNS *spp.* seen in this study in patients with chronic orofacial muscle pain was consistent with our previous observations (5) . In an investigation of staphylococcal nasal carriage in medical students, Kingdom et al. (20) showed that there were 291 CNS strains recovered from 197 students yielding a ratio of 12.7 strains for every 10 students studied. This colonisation prevalence of CNS was similar to the recovery frequency of 9 strains per 10 control subjects seen in this study. In a separate study (21), an attempt was made to determine the carriage of antibiotic-resistant CNS in patients with acne vulgaris. A total of 97 strains of CNS were recovered from the skin of 64 patients, giving a ratio of 15 strains for every 10 patients. In this study, where strict criteria were applied to prevalence, 23 strains of CNS were recovered from every 10 patients with chronic orofacial muscle pain, a colonisation prevalence nearly two fold higher than observed in the medical student study (20) and 1.6 fold higher than

Table 1 Colonisation incidence of coagulase-positive and coagulase-negative staphylococcal spp. from control subjects and patients with chronic muscle pain.

	Control subjects (n=8)	Chronic Pain patients (n=44)
Total staphylococcal spp.		
i) total incidence	11	114
ii) incidence/10 subjects	14	25
Coagulase positive staphylococcus (*Staph.aureus*)		
i) total incidence*	4	11
ii) incidence/10 subjects	5	3
Coagulase negative staphylococcus (CNS)		
i) total incidence*	7	103
ii) incidence/10 subjects	9	23
CNS		
Staph.epidermidis	3	32
Staph.warneri	0	12
Staph.haemolyticus	2	11
Staph.hominis	0	16
Staph.lugdunensis	0	14
Staph.xylosis	2	18

Significant Test: Chi square * = $P < 0.01$

Table 2. Incidences of control subjects (n=8) and chronic muscle pain patients (n=44) colonised with multiple staphylococcal carriage.

Number of CNS spp.	Control subjects (%)	Chronic muscle pain patients (%)
0	1	0
1	4	6 (13.6)
2	3	15 (34.1)
3	0	11 (25.0)
≥ 4	0	12 (27.3)

Significance Test: Chi Square ≥ 2 CNS spp $P < 0.008$
Fisher exact probability ≥ 3 CNS spp. $P < 0.006$

Table 3. Enterotoxins and TSST-1 production from control and muscle pain staphylococcal isolates.

	Control isolates		Muscle pain isolates	
Toxin	*Staph.aureus* (n=4)†	CNS (n=7)‡	*Staph.aureus* (n=11)†	CNS (n=103) ‡
Enterotoxin				
A	1	-	-	2
B	-	-	-	1
C	1	-	2	-
D	-	-	1	-
TSST-1	3	-	1	2
Total	5	0	4	5

Significance Test: Chi square with Yates corrections: † = Not Significant; ‡ = Not Significant.

Table 4. Incidence of positive and negative haemolysin from control (n=11) and muscle pain CNS isolates (n=114)

	Negative	Positive
Total staphylococcal isolates*		
Control (n=11)	9	2
Muscle pain (n=114)	54	60
Staph.aureus only †		
Control (n=4)	3	1
Muscle pain (n=11)	6	5
CNS only ‡		
Control (n=7)	6	1
Muscle pain (n=103)	39	64

Significance Tests. Chi square with Yates' correction: * = (P <0.03); † = NS; ‡ = (P<0.04)

Table 5. Incidence of staphylococcal α, β, and δ- haemolysins from control and muscle pain isolates

	Control isolates (n=11)		Muscle Pain isolates (n=114)	
Haemolysin	Negative	Positive	Negative	Positive
α*	10	1	104	10
β†	10	1	113	1
δ‡	11	0	73	41
'horse'¶	11	0	58	56

Significant Test: Chi square with Yates' correction: * = NS; † = NS
Fischer exact probability: ‡ = P < 0.04; ¶ = P<0.005
Positive haemolysis = % haemolysis greater or equal to 18.1% for α-haemolysis, 49.5% for β-haemolysis, 3.1% for δ-haemolysis, and 6.9% for 'horse'-haemolysin.

observed with the acne vulgaris study (21). This suggests that multiple carriage of CNS *spp.* is a significant and characteristic feature in patients with chronic orofacial muscle pain.

This study has shown that CNS are capable of producing TSST-1 and/or enterotoxins. The incidence of CNS from healthy individuals producing TSST-1 has previously been reported between 0.6% and 1.7% (22). Our data of 1.9% of CNS producing TSST-1 was in agreement with this finding. Despite the finding that CNS from chronic orofacial muscle pain patients can produce TSST-1, the low prevalence of these pyrogenic organisms is unlikely to cause significant pathology in this syndrome.

The ability of CNS to cause disease is now well accepted. CNS was the single most frequent organism recovered from neonates with necrotising enterocolitis (NEC) and was associated with both morbidity and mortality (23). The morbidity of NEC was shown to be significantly associated with patients colonised with CNS capable of producing cytotoxic amounts of δ-haemolysin (24,25). Gemmell (7) monitored the toxicogenic ability of commensal CNS from healthy individuals and found 13% of the strains produced δ-haemolysin. In this study 51.8% of CNS from patients with chronic orofacial muscle pain produced δ-haemolysin, a prevalence similar to the finding of Gemmell and Roberts (26) where 65% of CNS from patients with urinary tract infections, and 50% of CNS recovered from blood, abscess and wound infections produced δ-haemolysin. Interestingly chronic orofacial muscle pain patients report an increased prevalence of genitourinary infections, appendectomies and an increased prevalence of musculoskeletal problems in their long term partners (2). Similarly the age of increased prevalence of staphylococcal species associated urinary tract infections in healthy young females (27) also coincides with the increase in prevalence in reporting of orofacial muscle pain (28). These data suggest that patients with chronic orofacial muscle pain, although clinically non-infective, may have subclinical pathology occurring at cellular level resulting in reversible cell damage.

The data from this study indicate that CNS isolates producing α- and β- haemolysins were not significantly associated with chronic muscle pain patients, but δ-, and /or 'horse'- haemolysin producing strains were recovered from 89% of the chronic muscle pain patients and none of the control subjects. The *in-vivo* importance of δ-haemolysin as a virulent factor affecting host membranes has always been controversial. δ-haemolysin from CNS is a hexameric molecule of

25 amino acid residues with activities and a molecular homology to the 26 amino acids residues of δ-haemolysin from *Staph.aureus* (29,30). The activities of δ-haemolysin correlate directly with their ability to permeabilize cell membranes resulting in channel formation consisting of a cluster of α-helical molecules (31). The *in-vivo* roles of δ-haemolysin as a virulence factor has been challenged since serum lipoproteins and bactericidal lipids such as 2-monoglyerides and unsaturated fatty acids can neutralise δ-haemolysin activity (32,33). However, Long & Kapral (34) demonstrated the ability of CNS to produce fatty acid modifying enzymes (FAME) which inactivate both 2-monoglycerides and unsaturated fatty acids by esterifying the fatty acid moieties to cholesterol. Triglycerides with unsaturated fatty acid side chains produced by the host were shown to be potent inhibitors of FAME (35). Triglycerides in turn could be inactivated by bacterial lipase, a lipolytic exoenzyme, produced by CNS and other organisms (36). This series of measures and countermeasures of host-parasite relationship support the concept of host membrane permeabilisation by staphylococcal δ-haemolysin.

The wide spectrum of cytolytic activity of δ-haemolysin has been well reported. δ-haemolysin is known to lyse both human and horse red cells (37). The significant differences in the incidence of haemolytic activity shown in this study between human and horse red cells may confirm the broad but different cytolytic activity of δ-haemolysin. However, the result may also support the possibility of a different membrane damaging toxin from CNS; 'horse'-haemolysin. Turner and Pickard (38) reported a haemolysin from *Staph.aureus* which lyses horse red cells and with activities distinct from α-, β-, and δ-haemolysin. Not unlike δ-haemolysin 'horse'-haemolysin could withstand heating to 80^0C without loss of activity (38) a feature used in this study to highlight the probable presence of this new toxin. Furthermore, activities of 'horse'-haemolysis from each staphylococcal *spp*. observed in this study was uniformly and consistently different with the corresponding activities of δ-haemolysin providing further support for the concept of a different toxin. In view of the significant association of membrane damaging toxins from CNS and chronic orofacial muscle pain the identity and characteristics of this new 'horse'-haemolysin require urgent investigation.

The data from this study indicate that multiple carriage of CNS was associated with chronic orofacial muscle pain. The CNS recovered from 89% of the chronic muscle pain patients produced significant δ- and/or 'horse'-haemolysins, whereas those isolates from the musculoskeletal symptom free control subjects did not. It is proposed that CNS producing δ-, and/or 'horse'-haemolysins are highly associated with the aetiology of chronic orofacial muscle pain.

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